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Optimization of recombinant flavivirus antigens for infection serology:  
towards syndrome-based multiplex tests

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A Thesis submitted in fulfillment of the requirements of the Faculty of Life Sciences  
of The Open University (UK) for the Degree of Doctor of Philosophy

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Trieste, Italy  
January 2020



*Para Anunciacion Romero Luque  
Simplemente,  
¡GRACIAS!*



# ABSTRACT

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Sensitive and specific pathogen detection is an essential prerequisite for the prevention and treatment of infectious diseases. The similarities of clinical symptoms and serological cross-reactivity of viral structural antigens make the diagnosis of flavivirus infection problematic. Therefore, the main aim of this thesis was the development of a non-structural protein 1 (NS1) based serological assay for the diagnosis of flaviviruses.

Recombinant NS1 (rNS1) oligomers consistent with the native secreted form of the protein were purified for TBEV, WNV, ZIKV, USUV, and DENV 1-4. The ability of rNS1 proteins to detect specific antibodies was analyzed using sera of immunized mice and well-characterized human sera samples. These antigens were used in a standard ELISA format and shown to be highly sensitive and specific compared to commercial assays.

The optimized NS1-based ELISA was used to assess the IgM/IgG responses to WNV and USUV in North-Eastern Italy. The results of the analysis confirmed the area as endemic for USUV and contributed to the characterization of the first human cases of USUV infection in blood donors. The NS1-based ELISA was also applied to 200 sera samples from patients exhibiting febrile illness who visited the University of Maiduguri Teaching Hospital in Nigeria. Only 11 of 200 serum samples were negative for all the flaviviruses tested, while all other samples were positive for at least one pathogen. Molecular analysis confirmed the circulation of flaviviruses in the region, including zika virus.

In conclusion, rNS1 represents a valuable option for the serology of flaviviruses with reduced cross-reactivity and high sensitivity.



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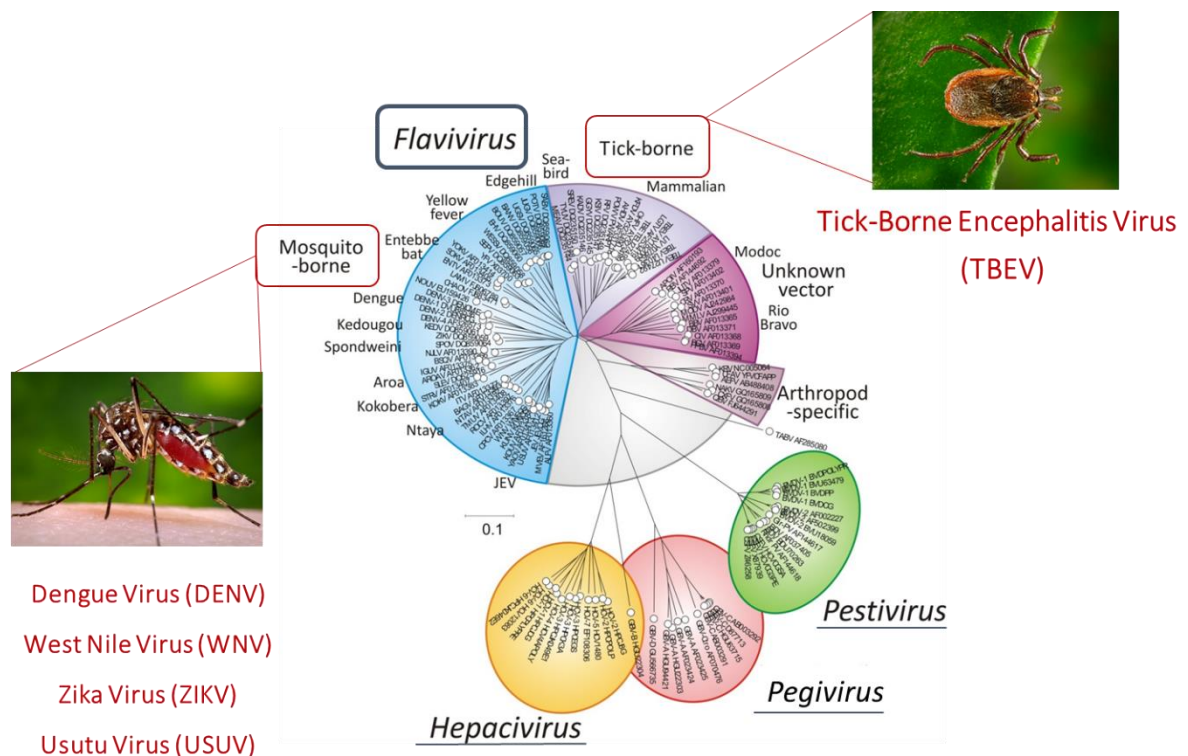
# INTRODUCTION

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## 1. INTRODUCTION

### 1.1. Flavivirus – an overview

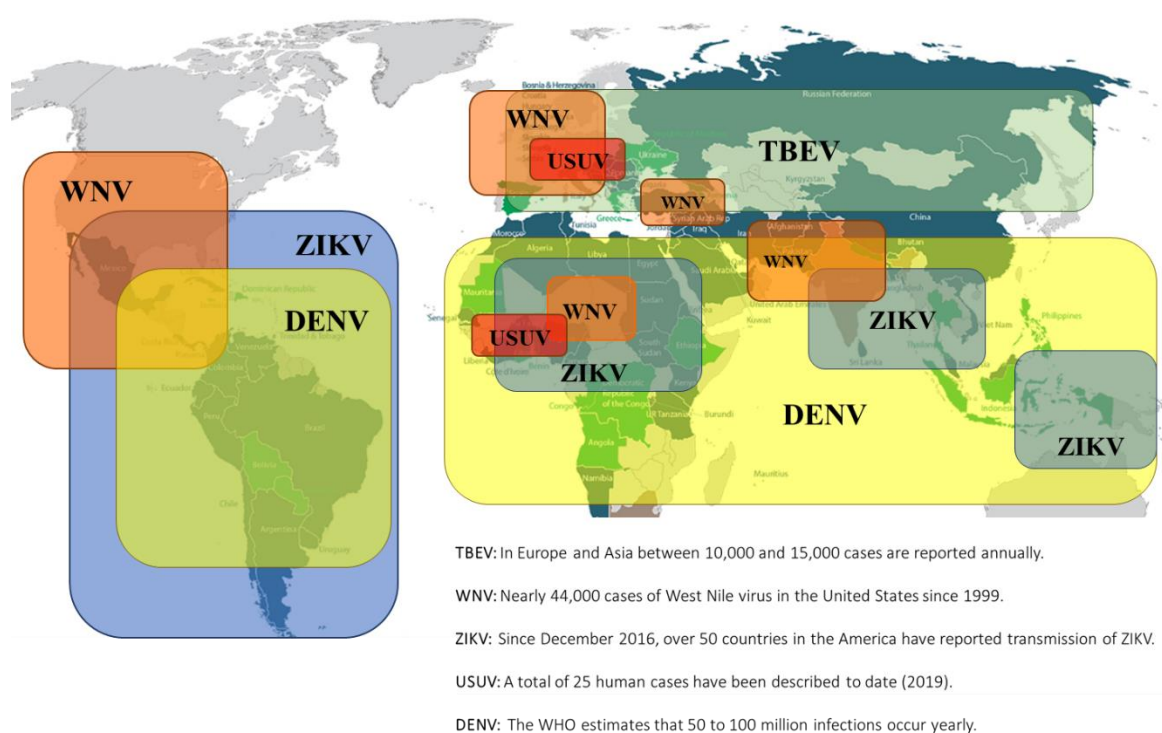
Flavivirus is a *genus* of small, arthropod-borne, enveloped viruses containing a single positive-strand genomic RNA of approximately 11kb that belongs to the family *Flaviviridae* (Gubler et al. 2017). This family of viruses is composed of a large group of important human and veterinary pathogens classified in four different *genera* (Figure 1.1): hepacivirus, flavivirus, pestivirus, and pegivirus (Simmonds et al. 2017). They are grouped based on conserved motifs of the RNA-dependent RNA polymerase (RdRp). All flaviviruses share standard genome organization and structural characteristics; however, the biological properties of each group are different (Schweitzer et al. 2009; Simmonds et al. 2017).



**Figure 1.1.** Phylogenetic tree of conserved amino acid sequences of several members of the family *Flaviviridae*. The tree was constructed with the sequence alignment of representative isolates of different members of the family *Flaviviridae* by neighbor-joining. Relevant mosquito and tick-borne flaviviruses included in this thesis are shown (Adapted from the International Committee on Taxonomy of Viruses, Ninth Report 2011).

Flaviviruses are the most prevalent arthropod-borne viruses worldwide and comprise over 70 different antigenically related members. The wide range of vectors and the ability of the vectors to infect rodents, pigs, non-human primates, humans, and other mammalian hosts, explain the burden and the distribution of flaviviruses in all the continents (Figure 1.2) (Gupta et al. 2014; Daep et al. 2014). Among critical human pathogens, tick-borne encephalitis virus

(TBEV), west nile virus (WNV), zika virus (ZIKV), dengue virus (DENV), yellow fever virus (YFV) and japanese encephalitis virus (JEV) are considered to affect millions of people with an increasing spread in non-endemic areas where the mosquitos are present (Gubler 2002; Woolhouse et al. 2012). Besides, re-emerging flaviviruses such as usutu virus (USUV) are affecting new areas where few cases were reported in the past (Lim et al. 2018; Roesch et al. 2019; Zannoli and Sambri 2019).



**Figure 1.2. Map of the global distribution of flaviviruses.** The areas where human cases of infection with flavivirus have been reported are shown. Tick-borne encephalitis virus (TBEV, green), west nile virus (WNV, orange), zika virus (ZIKV, blue), usutu virus (USUV, red) and dengue virus (DENV, yellow). Additionally, some information demonstrating the impact of flaviviruses is shown.

Clinical manifestations upon infection with flaviviruses can range from undifferentiated fever and mild symptoms to more severe presentations that can potentially lead to death. Secondary DENV infections has been well-documented to cause hemorrhagic fever; while, infections with neurotropic viruses such as ZIKV, YFV, WNV, TBEV, and JEV are responsible for many viral encephalitides in the world (Johnson 2016; Peltier et al. 2013). The infection with these viruses can cause severe damage to the nervous system that can lead to the development of neurological complications such as Guillain-Barre syndrome, microcephaly, and meningitis (Cobo 2016; Gubler 1998, Oliveira Melo et al. 2016; Turtle, Griffiths, and Solomon 2012).

Many flaviviruses of human public health significance are mainly transmitted between mammalian hosts by *Aedes* mosquitoes and *Ixodes* ticks (Gubler et al. 2017; Kuno and Chang 2005). The virus can replicate in both the vertebrate host and the vector. Arthropods are usually infected once an adult female mosquito bites a viremic human, acquiring the circulating virus along with the blood meal. In the vector, the virus passes through the midgut barrier, and viral dissemination occurs through hemolymph to secondary target organs until finally reaching the mosquito salivary glands. The infected mosquito can also transmit the virus by biting a vertebrate host (Hegde, Rasgon, and Hughes, 2015). Following the bite, the virus replicates and spreads to other organs through the lymphatic system and the bloodstream (viremic phase). When a viremic host is bitten by a susceptible vector, the virus infection cycle continues (Carrington and Simmons 2014).

Arbovirus transmission allows crossing species barriers since the same arthropod may bite birds, reptiles, and mammals that rarely are in contact with one another naturally (Parrish et al. 2008). Flaviviruses have three overlapping transmission cycles: a sylvatic cycle, a rural cycle that occurs in “emergence zones,” and an urban cycle. The sylvatic flavivirus transmission cycle involves an arthropod vector and a non-human vertebrate reservoir (birds, rodents, bats, or primates). Except in the case of dengue and yellow fever, humans are not involved in the primary transmission cycles. Humans are infected as an accidental event when entering into this natural ecosystem. The urban cycle utilizes domesticated mosquitoes and humans as hosts (Vasilakis and Weaver 2017). Long-distance air travels, rapid urbanization, widespread deforestation together with the possible adaptation of flavivirus to new habitats and host species have also contributed to the expansion of the pathogens into previously non-endemic areas (Bhatt et al. 2013; Petersen and Marfin, 2003).

Extensive work has been dedicated to developing potential anti-flavivirus therapeutics and treatments that can improve the care of infected individuals, especially in healthcare resource-poor countries. However, they are not commercially available yet (Sun, Chen, and Lai, 2018; Wilder-smith et al. 2018; Zakaria, Carletti, and Marcello, 2018). Flavivirus infections are managed with rest and supportive therapy based on the clinical presentations without hospitalization. Vaccination is considered the most vital intervention to prevent flavivirus infections. Effective inactivated or live-attenuated whole virus vaccines against yellow fever, Japanese encephalitis, and tick-borne encephalitis infections are available, but human vaccines

against other flaviviruses such as WNV, DENV, and ZIKV are still in development (Collins, Metz, and Carolina 2017; Heinz and Stiasny 2012; Wilder-smith et al. 2018).

The major interest in this introduction is focused on tick-borne encephalitis virus, west nile virus, zika virus, usutu virus, and all four dengue virus serotypes.

### **1.1.1. Tick-borne encephalitis virus**

Tick-borne encephalitis virus (TBEV) is an endemic virus in many European countries and in central and eastern parts of Asia, which corresponds to the distribution of the arthropod vectors (Carletti, Zakaria, and Marcello 2017; Hayasaka et al. 2001; Schotthoefer 2015). Based on their genomic sequences, TBEV can be classified into three subtypes: European (Eu), Siberian (Sib), and Far Eastern (FE). Although there are small variations (2-3%) between TBEV subtypes at the amino acid level, they present different clinical manifestations. An infection with Sib-TBEV and FE-TBEV is usually identified for the development of influenza-like illness. Meanwhile, an infection caused by Eu-TBE is characterized by a biphasic disease after 1-2 weeks. Patients in the first phase present headache, fever, malaise, and muscle pains followed a symptom-free period of approximately seven days. During the second phase, about one-fourth of the patients develop neurological symptoms (Mandl 2005).

Transmission of TBEV occurs when a person is bitten by an infected tick, by consuming unpasteurized milk and cheese or dairy products from TBEV infected animals (Lindquist and Vapalahti 2008; Offerdahl et al. 2016). Transmission by blood transfusion has not been documented but may be an additional risk (Caracciolo, Carletti, 2015). The main arthropod vectors involved in TBEV dissemination are ticks of the species complex *Ixodes ricinus* and *Ixodes persulcatus*, that act both as the vectors and the reservoir of TBEV. In Europe, TBEV represents the most important viral disease transmitted by the tick vector (Süss 2011). Annually, from 10.000 to 15.000 TBEV infection cases are reported. To date, no drugs or specific treatments are approved for TBEV, and despite the availability of efficient purified inactivated whole virus vaccines, the incidence of TBEV is on the rise as vaccine coverage is insufficient for many risk groups (Kunze 2012).

### **1.1.2. West Nile virus**

West nile virus (WNV) is also a significant health concern for humans. Of all the mosquito-borne flaviviruses, WNV has the most widespread geographical distribution and the largest

vector and host range (Blitvich and Bradley, 2008). WNV infection was reported for the first time in 1937 from a febrile illness patient in Uganda (Smithburn et al., 1940). Subsequently, the frequency, severity, and geographic range of WNV outbreak increased, and outbreaks of WNV meningitis and encephalitis affecting primarily adults have been reported in Africa, Europe, the Middle East, North America and West Asia (Chancey et al. 2015). The potential risk of WNV propagation in other regions has been suggested since a few human cases in Central and South America are found in the literature (Komar and Clark 2015).

WNV is naturally maintained in an enzootic cycle between birds and mosquitoes. In particular, birds play an important role in virus spreading, while mosquitos are responsible for the accidental infection of humans and horses, the dead-end hosts of the virus (Weissenböck et al. 2010). Although there is much effort to reduce the risk of WNV human infections, WNV transmission has currently been reported in several countries in Europe. According to the ECDC, since the beginning of the 2019 to 3 October 2019, 404 WNV human infections have reported in EU member states and EU neighboring countries. Among them, 39 deaths due to west nile virus infection have been reported (ECDC, 3 October 2019). Approximately 80% of WNV infections in humans have no symptoms, and a small percentage of infected patients present fever some days after the onset of the symptoms. Importantly, around 1% of individuals infected with west nile virus will develop a more severe form of disease leading to west nile neuroinvasive disease, such as west nile encephalitis or meningitis or west nile poliomyelitis (Sejvar 2014).

### **1.1.3. Zika virus**

ZIKV was isolated for the first time from a febrile monkey in Uganda (Dick et al. 1952). The identification of the first human case of ZIKV is unclear; it was identified in Africa, possibly in Nigeria 1954 or Uganda 1962 (Macnamara et al. 1954; Gelfand et al. 1964).

For over half a century, a few reports describing clinical presentations similar to other flavivirus infections in Africa and Asia indicate the circulation of ZIKV. However, it was not until 2007 and 2013, when two main outbreaks of ZIKV were reported in Yap Island Micronesia and French Polynesia, respectively (Kindhauser et al. 2016). The biggest outbreak of ZIKV was reported in Brazil in 2015 (Zanluca et al. 2015). After that, autochthonous infections with ZIKV have been reported in more than 87 countries an territories, including 13 from Africa, 49 from Latin America, 6 from South-East, and 19 from Asia Western Pacific region (WHO, 2019).

Although *Aedes albopictus* mosquitos are present in Southern Europe, autochthonous cases have not been reported yet (Spiteri et al. 2017).

Comparable to other flavivirus infections, ZIKV is transmitted by *Aedes* mosquitos, and most infected people develop mild symptoms without needing hospitalization. However, some complications of zika infection can be associated with the development of some neurologic disorders. Importantly, ZIKV infection during pregnancy have called significant attention due to the development of microcephaly in newborns (Tetro 2016; Mlakar et al. 2016) and Guillain-Barré syndrome in adults (Barbi et al. 2018), as well as, due to transmission from the mother to the fetus (Mysorekar 2017), organ transplantation (Nogueira et al. 2016) sexual transmission (Musso et al. 2015) and possibly by blood transfusion (Magnus et al. 2018). Added to this, antiviral treatment, and vaccines against ZIKV are not clinically available.

#### **1.1.4. Usutu virus**

Usutu virus (USUV) is a mosquito-borne flavivirus that was first identified and isolated in 1959 from *Culex neavei* mosquitos in South Africa and is maintained through an enzootic cycle involving birds as the main amplifying reservoir hosts and *Culex* mosquitos as vectors (Gaibani and Rossini 2017).

The first case of USUV infection in humans was diagnosed at the beginning of 1980 in the Central African Republic in a patient with fever and rash, while the second case was a 10-years-old patient with fever and jaundice identified in Burkina Faso in 2004 (Nikolay et al. 2011). Since then, several African countries, including Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Cote d'Ivoire, and Morocco, have reported the circulation of USUV (Nikolay et al. 2011). Even though avian infections were not reported to be fatal in Africa, USUV emerged in Europa in 1996, causing high numbers of bird deaths. In 2001 USUV caused a significant number of deaths in blackbirds and great grey owls in Vienna, Austria, and later spread to Spain, Hungary, Italy, Switzerland, and Germany (Lühken et al. 2017; Nikolay et al. 2011; Weissenböck et al. 2003).

In 2009, the first human cases of USUV infection in Europa were reported in Italy, causing meningoencephalitis in two immunocompromised patients (Pecorari et al. 2009; Cavrini et al. 2009). Including these two cases, a total of 25 documented human cases of USUV virus infections have been reported in Europa until June 2019 (Zannoli and Sambri 2019). Some of

them characterized for neuroinvasive infection (Gaibani and Rossini 2017; Lühken et al. 2017; Simonin et al. 2018).

Frequently, USUV co-circulates with west Nile virus in many European countries, in terms of the geographic range of transmission, host and vector species. By using modeling approaches, a study performed in 2018 demonstrated that large areas in Europe are suitable for USUV infection and represent a high risk of re-emerging pathogens like USUV in Europe (Cheng et al. 2018). However, surveillance studies to analyze the incidence of USUV in humans are lacking. It results in a few documented USUV cases compared to the high incidence of WNV (Calzolari et al. 2010).

#### **1.1.5. Dengue virus**

Dengue is a systemic viral infection transmitted by mosquitoes infected with dengue virus (DENV). DENV infectious have increased drastically during the last 50 years, some studies have reported that more than 390 million infections occur every year, among them, 90 million are characterized by asymptomatic patients (Bhatt et al. 2013). DENV has a worldwide distribution, and it correlates to the presence of *Aedes aegypti* mosquito predominantly in the tropics and subtropics. Also, the spread of *Aedes albopictus* to North America and more than 25 countries in the European Region fuelled by viremic travelers, put temperate areas such as Europe at risk for transmission of DENV and other *Aedes* transmitted viruses (Ryan et al. 2019; Massad et al. 2018).

Four genetically related but antigenically distinct dengue virus serotypes (DENV1-4) have been described. They have been confirmed by nucleic acid sequencing and ecologic, phylogenetic, and evolution studies (Holmes and Twiddy 2003; Weaver and Vasilakis 2009). Because of the high genetic similitudes between all four DENV serotypes (approximately 65%), the infection with a specific serotype induces a life-long protective immunity to the homologous serotype, but secondary infection with heterologous serotype is more severe, mainly due to the development of dengue hemorrhagic fever and dengue shock syndrome (Alejandria 2015; Ranjit and Kissoon 2011). It has been suspected that antibody-dependent enhancement (ADE) of dengue virus growth occurs when the presence of preexisting antibodies binds to an infecting DENV particle during secondary infection with a different serotype or from low concentrations of dengue antibodies of maternal origin in infant



sera. These non-neutralizing antibodies form an antibody-virus complex that facilitates virus entry into host cells, leading to increased infectivity in the cells (Katzelnick et al. 2017).

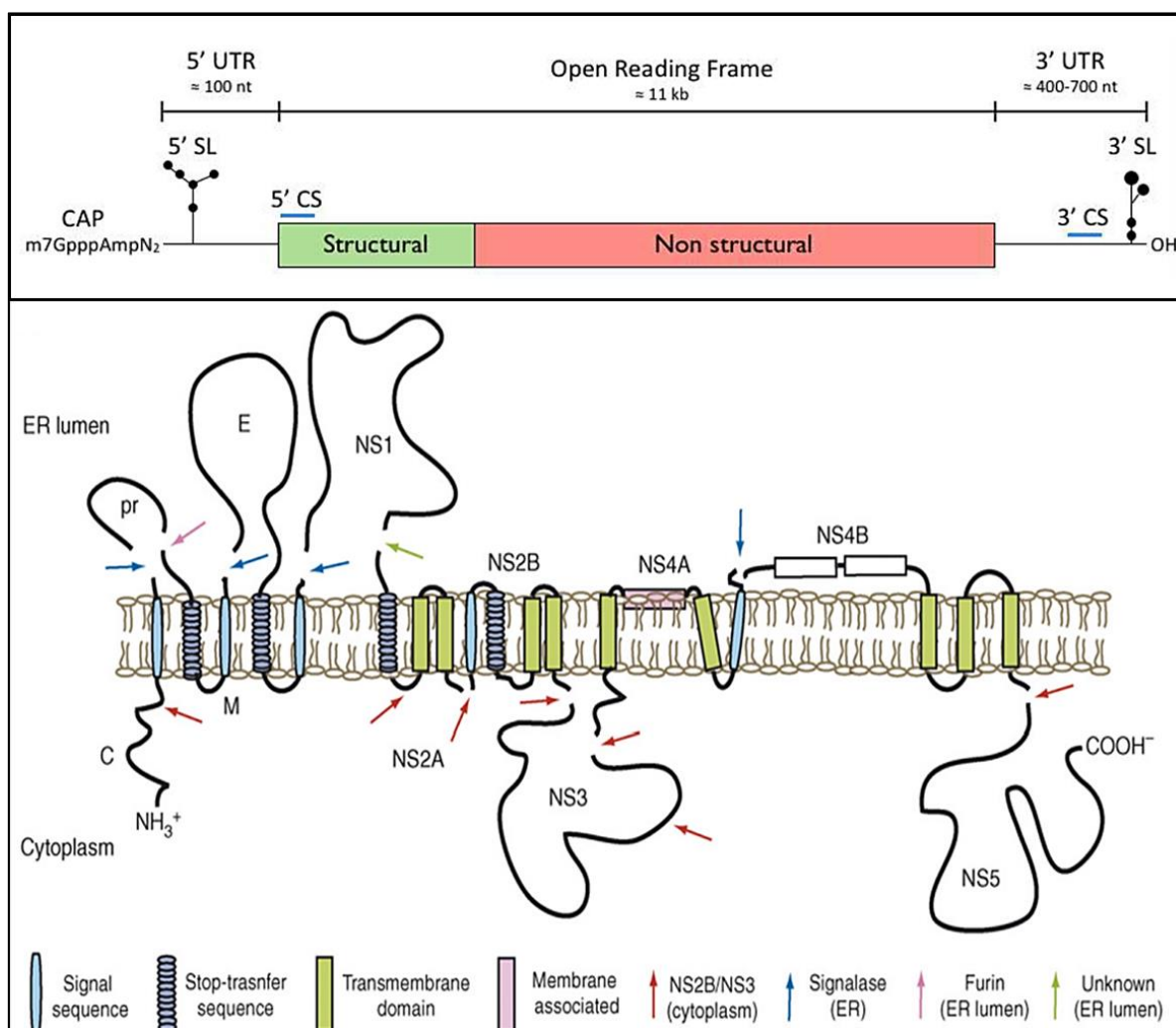
Despite the considerable amount of work made over the years, there are no effective antiviral agents to treat dengue infection, and the treatment, therefore, remains supportive (Wiwanitkit 2010). The development of an efficient vaccine against the dengue virus is still unresolved and faces many challenges. The most advanced dengue vaccine was registered in 2015, Dengvaxia (CYD-TDV) by Sanofi Pasteur. The CYD-TDV is a live attenuated dengue vaccine that expresses the envelope and pre-membrane proteins of all dengue virus serotypes and the yellow fever virus (strain 17D) nonstructural proteins, including NS1 (Guy et al. 2011; Guy and Jackson 2015). So far, the CYD-TDV vaccine has been authorized in 19 countries, mainly in Latin America and Asian countries. However, the high incidence of hospitalizations in DENV naïve children who received the vaccine makes its future unclear (Anderson, Endy, and Thomas 2018).

## **1.2. Genomic organization of flaviviruses**

Flaviviruses are enveloped icosahedral viruses of about 50 nm in diameter and contain a positive-sense single-stranded RNA genome of ~11 kb in length. The genome is composed of one open reading frame (ORF) flanked by two untranslated regions (UTR) that interact with viral and host proteins (Figure 1.3). The 5'UTR region is characterized by a type I cap (m7GpppAmpN2) as a product of the methyltransferase and nucleotide triphosphatase activity of NS5 and NS3, respectively (Mazeaud et al. 2018; Wang et al. 2018). The 3'UTR region lacks the typical polyadenylated tail of cellular messenger RNAs, and it is composed of three regions that are implicated in viral adaptation to the host (Mazeaud et al. 2018; Villordo et al. 2015). Both 5'UTR and 3'UTR contain sequences motifs that are involved in viral RNA translation, replication, and possibly packaging (Mazeaud et al. 2018; Selisko et al. 2014). The genome is translated directly from the RNA genome as a single polyprotein, and subsequently it is cleaved by both viral and host proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Mazeaud et al. 2018).

The polyprotein is integrated into the endoplasmic reticulum (ER) membrane. Structural proteins and the NS1 are mainly situated in the lumen of the ER, while the NS3 and NS5 are present in the cytoplasm site. The others are transmembrane proteins that can be found on both sides of the ER membrane (Fernandez-Garcia et al. 2009; Perera and Kuhn 2008). The structural proteins provide the icosahedral shape, including the envelope protein that is

involved in receptor-mediated endocytosis, endosome fusion, and release of the virion (Zhang et al. 2017). While the polymerase and protease functions of flavivirus nonstructural proteins are essential for virus replication and assembly (Murray, Jones, and Rice 2009).



**Figure 1.3. Schematic diagram of Flavivirus polyprotein organization and processing.** Top panel: representation of the flavivirus genome of approximately 11 kb. Simplified open reading frame showing RNA secondary and tertiary structures within the untranslated regions (UTRs) is indicated. CS, cyclization sequence; VR, variable region; SL, stem-loop. Bottom panel: schematic representation of the organization of structural and nonstructural proteins in the endoplasmic reticulum (ER) membrane predicted from biochemical and cellular analyses, which is then processed by cellular and viral proteases, indicated by arrows (adapted from Fernandez-Garcia et al. 2009; Perera and Kuhn 2008).

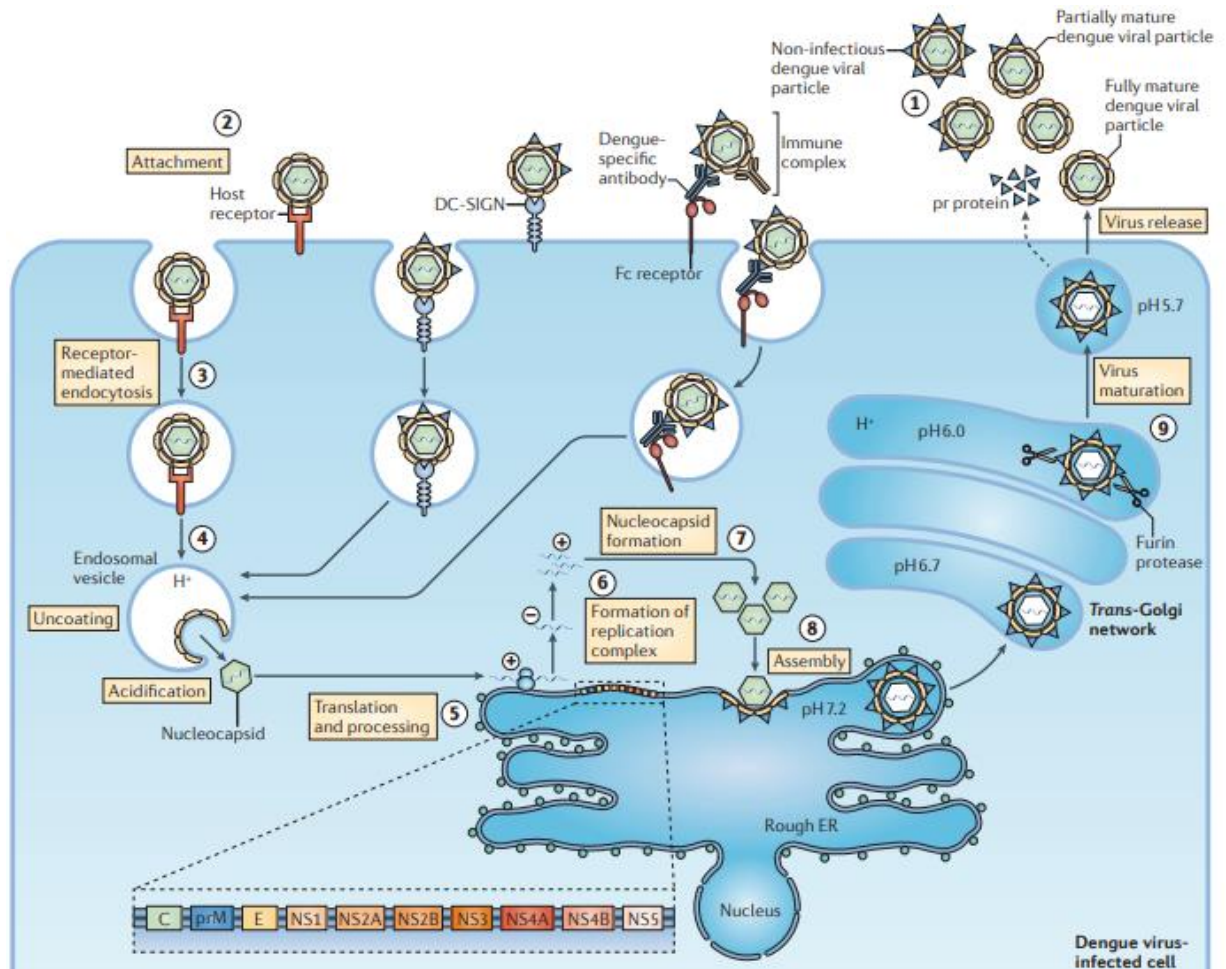
### 1.3. The life cycle of flaviviruses

The general process during the life cycle of flaviviruses is shown in figure 1.4. It involves all the steps from the attachment of the virus to the cell surface to the packaging of virions and the release of the new progeny (Screaton et al. 2015).

Flavivirus entry into the host cell occurs through interactions between the envelope protein and cell surface receptors. These interactions facilitate receptor recognition and binding that leads to receptor-mediated endocytosis (Schaar et al. 2008). Due to the low pH in the endosome, the envelope proteins present in the outer face of flavivirus particles suffer irreversible trimerization that results in the exposure of a fusion peptide (Martín, Liu, and Kielian 2010). This peptide allows the fusion and release of the nucleocapsid into the cytoplasm (Allison et al. 1995; Modis et al. 2004).

After uncoating of the nucleocapsid, the viral RNA is released into the cytoplasm. The genome has three different functions: replication, translation, and association with nascent viral particles. Flavivirus replication occurs in close association with virus-induced membrane structures of the rough ER, predominantly in the perinuclear region. These membrane structures are clusters of about 100 nm vesicles, called vesicles packets that may serve as a scaffold for anchoring the replication complex, or to limit the diffusion of viral/host proteins and viral RNA increasing the concentration of components required for RNA synthesis (Miller and Krijnse-locker 2008; Miorin et al. 2013; Paul and Bartenschlager 2013; Sreaton et al. 2015).

Apart from the genome that has been replicated and translated as a single polyprotein, the newly synthesized genomic RNA interacts with capsid protein localized on the cytoplasmic side of the ER to form the nucleocapsid that buds into the ER membrane to acquire the prM and envelope proteins. This leads to the budding of immature virions into the ER. These virions are subsequently transported through the secretory pathway, where glycans on prM and envelope proteins are modified. The last step of virion maturation involves furin-like protease-mediated cleavage of prM protein that occurs during egress to generate mature infectious viruses. Depending on the level of PrM cleavage, fully mature, entirely immature and partially mature particles can be produced (Fernandez-Garcia et al. 2009; Mukhopadhyay, Kuhn, and Rossmann 2005; Sreaton et al. 2015).



**Figure 1.4. Schematic representation of the general steps involved in the flavivirus life cycle.** (1) Depending on the level of PrM cleavage, viral particles exist as a mixture of different structures: fully mature particles, entirely immature particles, and partially mature particles. (2). The virus attaches to the host cell receptor and is internalized by receptor-mediated endocytosis and trafficked to early endosomes. (3). Low pH in the endosome induces fusion between the virus and the host membrane resulting in genome release. (4). The viral ARN is translated into a single polyprotein that is cleaved by viral and host proteases (5) before commencing the replication process (6). Packaging of newly synthesized RNA genomes occurs on the surface of the ER (7). Viral assembly initiates when the nucleocapsid buds into the ER (8). The immature virions are transported to the trans-Golgi, where Furin-mediated cleavage of prM to M generates mature infectious particles that are released by exocytosis (9) (Adapted from Screaton et al. 2015).

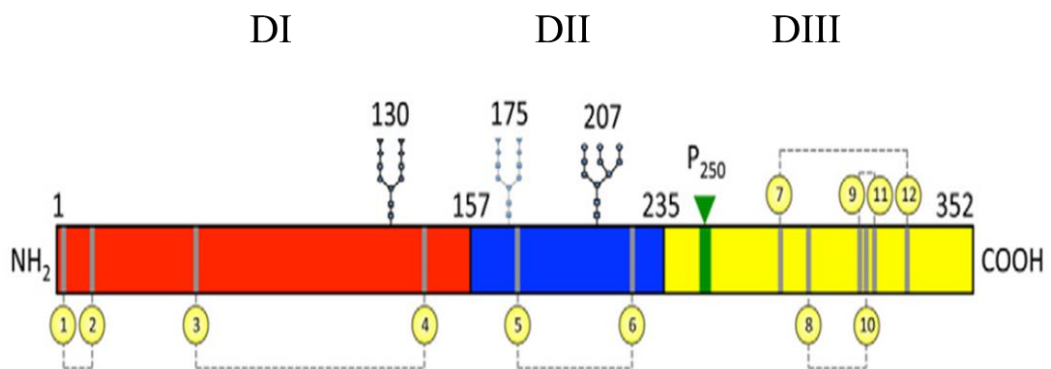
#### 1.4. Flavivirus viral proteins

As mentioned above, the flavivirus genome is translated as a single polyprotein that is subsequently cleaved in 3 structural and 7 nonstructural proteins. Each protein plays an essential role in the life cycle of flavivirus. However, this thesis is focused on NS1, a protein that plays several roles in the life cycle of flaviviruses. NS1 is also found at high levels in blood circulation in infected patients. It has opened the possibility to use NS1 as a target for the development of vaccines and diagnostic tests.

### 1.5. The nonstructural protein 1 (NS1)

The nonstructural protein 1 (NS1) was reported for the first time in 1970 as a non-hemagglutinating, soluble complement-fixing antigen in the serum and brain extracts of infected mice with dengue virus 2 (Brandt, Cardiff, and Russell 1970; Russell, Chiewsilp, and Brandt 1970). In 1985 the antigen was renamed NS1 after the sequencing of the yellow fever genome (Rice et al. 1985). NS1 genes are highly homologous among the NS1 sequences of different flaviviruses and regions of similarity and dissimilarity has been confirmed by using multiple alignment tools (Rastogi, Sharma, and Singh 2016).

As shown in figure 1.5, flavivirus NS1 genes consist of 1056 nucleotides in length, encoding a 352-amino-acid polypeptide with one, two or three (depending on the virus) N-linked glycosylation sites (positions 130, 175 and 207) and 12 conserved cysteine residues that form 6 intra-chain disulfide bonds (Blitvich et al. 2001; Mandl, Heinz, and Kunz 2004). Using mass spectrometry has been demonstrated the arrangement between all 6 disulfide bonds of DENV NS1 proteins (Blitvich et al. 2001; Wallis et al. 2004). These disulfide bonds (C1-C2, C3-C4, C5-C6, C7-C12, C8-C10, and C9-C11) play an essential role in the proper folding of NS1 monomer, dimerization, secretion, and oligomer formation, as demonstrated by the substitution of Ala for Cys residues present in NS1 (Pryor and wright 1993; Winkler et al. 1989).



**Figure. 1.5. Linear representation of secreted NS1 protein.** Secreted NS1 protein is formed by three different domains: Domain I (red), Domain II (blue), and Domain III (yellow). 12 conserved disulfide bonds between the NS1 monomer of all flaviviruses are indicated with yellow spheres. Glycosylation sites at positions 130, 175, and 207 are also represented. The glycosylation site at position 175 (shown in grey) is only conserved between viruses belonging to the Japanese encephalitis serocomplex (Adapted from Muller and Young 2013).

All the steps from the synthesis to the secretion of NS1 are summarized in figure 1.7 and described below with more detail as follow:

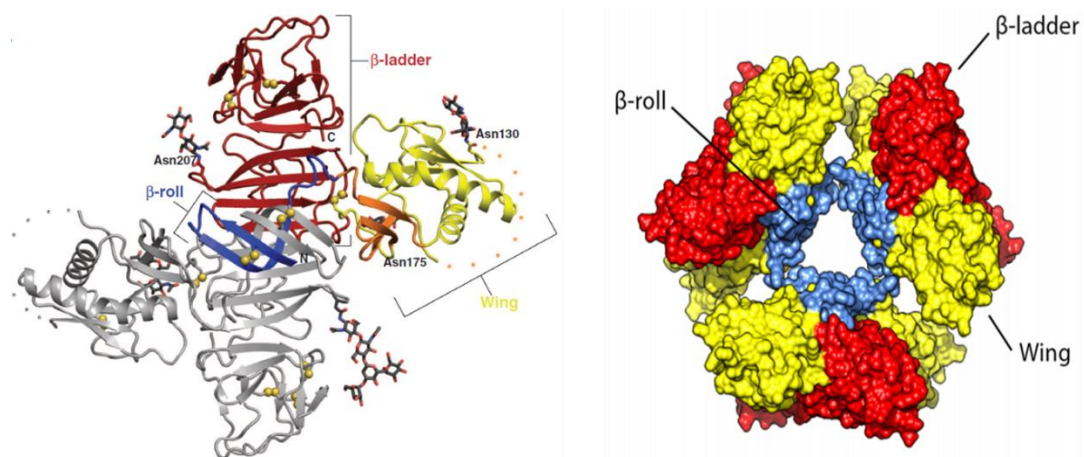
#### **1.5.1. Translation and cleavage of NS1**

The NS1 protein is synthesized at the endoplasmic reticulum (ER), and then, it is translocated in the lumen of the ER due to the presence of a signal sequence encoded by the last 24 amino acids of the preceding envelope protein (Falgout, Chanock, and Lai 1989). During its synthesis in infected cells, the hydrophilic NS1 monomer is cleaved from envelope protein by the ER-resident host signal peptidase (Nowak et al. 1989). Meanwhile, the cleavage at the C-terminus of NS1-NS2A requires a minimum length of eight amino acids (L/M-V-X-S-X-V-X-A), which is critical for cleavage by a host membrane-bound ER-resident host protease, possibly signalase (Falgout and Markoff 1995).

#### **1.5.2. Structure of NS1**

As is shown in figure 1.6, the X-ray crystallographic analysis of WNV and DENV-2 has revealed that each NS1 monomer has three separate structural domains: the hydrophobic  $\beta$ -roll dimerization domain (amino acids 1–29), followed by an  $\alpha/\beta$  wing domain (amino acids 30–180) which contains two glycosylation sites (Asn130 and Asn175), and finally the predominant structural feature of NS1 is the third domain, a continuous  $\beta$ -ladder domain (amino acid residues 181–352) containing one glycosylation site (Asn 207) and an extended  $\beta$ -sheet on one face and a “spaghetti loop” on the opposite face (Akey et al. 2014; Xu et al. 2016).

Newly synthesized NS1 appears as a monomer, but around 20-30 minutes after synthesis, NS1 forms a detergent-resistant dimer that is sensitive to heat and low pH treatment (pH 2.2-3) (Winkler et al. 1989). The NS1 dimer is formed when two  $\beta$ -roll domains dimerize at the center, and these dimers tend to trimerize, resulting in hexameric NS1 (Edeling, Diamond, and Fremont 2014; Gutsche et al. 2011). When the NS1 dimer is assembled, two different faces are formed. The “inner face” is hydrophobic and displays the  $\beta$ -roll domain and the exposed  $\beta$ -sheet from the central  $\beta$ -ladder. The “outer face” is hydrophilic and displays the glycosylation sites and the spaghetti loop present in the  $\beta$ -ladder domain (Akey et al. 2016; Xu et al. 2016).



**Figure 1.6. Graphic representation of NS1 structure.** The left panel shows the NS1 dimer with one subunit in gray and the other colored by the domain (blue,  $\beta$  roll domain; yellow, the wing domain with orange connector subdomain; red, central  $\beta$  ladder domain). Disulfides are shown as yellow spheres and N-linked glycosylation sites as black sticks. The right panel shows the 3D organization of the NS1 hexamer, with NS1 domains highlighted in the same color-code of the dimer (Adapted from Akey et al. 2014; Scaturro et al. 2015).

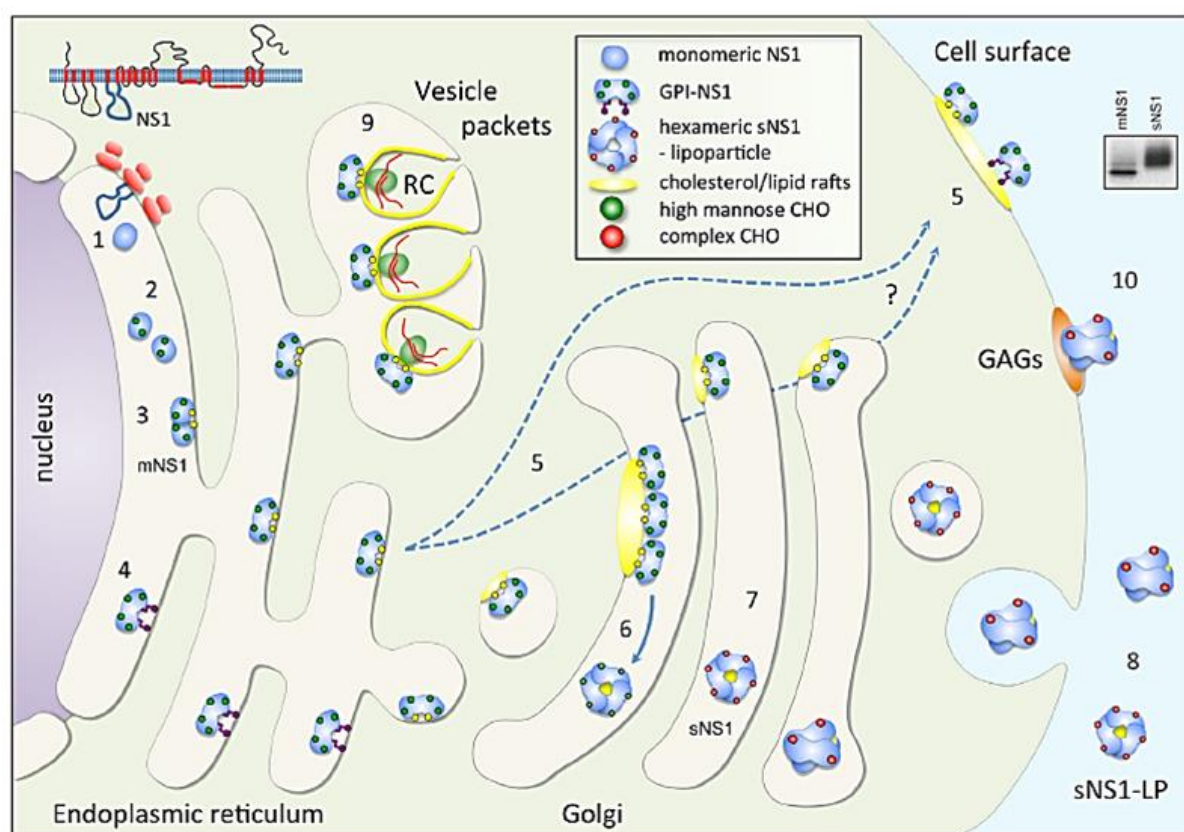
### 1.5.3. Cellular location of NS1

NS1 can be found in multiple forms, and the functions are closely associated with its cellular location throughout the virus replication cycle (Figure 1.7). NS1 is mainly present as a membrane-associated dimer in the luminal side of the ER, where it is trafficked to the site of RNA replication in vesicle packets (Falgout and Markoff 1995). In addition to remaining within the ER, intracellular dimeric NS1 is trafficked to the cell surface, by an unidentified mechanism that requires charged amino acids near the N-terminus of NS1 to allow the association of NS1 with intracellular membranes (Youn et al. 2010). Flavivirus NS1 does not contain a transmembrane domain, and there is no evidence for posttranslational protein modification that can explain its affinity for the membrane. Only for DENV have been identified a glycosylphosphatidylinositol (GPI)-linked form of NS1, which is produced in the ER and allows binding to internal membranes and the outer face of the plasma membrane (Jacobs et al. 2000; Noisakran et al. 2007, 2008).

The  $\beta$ -roll domains of NS1 dimer have a partially hydrophobic nature, and therefore, it could explain the association of the protein with ER membranes (Akey et al. 2014). In addition, analysis based on site-directed mutagenesis has demonstrated that the dimerization step is a prerequisite for NS1 transport to the cell surface and secretion from infected cells. It has been proved by the substitution of alanine for any of the last three cysteine residues, which are located within the carboxy-terminal amino acid sequence of the protein (Pryor and wright



1993). In addition, the crystal structure of full-length ZIKV NS1 revealed a long-intertwined loop forming a hydrophobic “spike” in the wing domain that can contribute to the membrane association together with the  $\beta$ -roll domain. However sequence analysis reveals that the electrostatic potential of the outer surface of ZIKV NS1 is markedly different between different flaviviruses (Brown et al. 2016; Song et al. 2016; Xu et al. 2016).



**Figure 1.7. NS1 processing and trafficking in mammalian cells.** (1) NS1 synthesized at the ER is translocated to the lumen of the ER due to the presence of a signal sequence in the C-terminus of the preceding envelope protein. (2) NS1 is present in the ER as a monomer, which is modified by the addition of high mannose carbohydrates at 2 or 3 sites (depending on the virus). (3) Following glycosylation, NS1 rapidly dimerizes and becomes membrane associated due to an acquired hydrophobic nature. (4) NS1 is trafficked to 3 distinct locations in infected cells: (5) a) membrane-associated NS1 is trafficked to the cell surface by an unidentified pathway. (6) b) a subset of NS1 traffics through the Golgi, where the dimers associate to form a soluble hexamer. (7) Here the high mannose carbohydrates are processed to more complex sugars, and (8) the soluble hexamer is subsequently secreted to the extracellular fluid. (9) NS1 is also trafficked to the site of RNA replication in vesicle packets, where it associates with other components of the viral replication complex. (10) Secreted NS1 can bind back to infected and uninfected cells by association with glycosaminoglycans (adapted from Muller and Young 2013).



#### 1.5.4. Hexameric NS1 formation and secretion

NS1 is transported through the Golgi pathway and secreted as higher-order oligomers, including hexamers in the extracellular medium from infected mammalian and insect cells (Alcalá, Palomares, and Ludert 2018; Flamand et al. 1999; Muller and Young 2013; Winkler et al. 1989). Based on WNV, the secretory profile of flavivirus NS1 can be regulated by two N-terminal amino acids (10 and 11) that directs NS1 to the plasma membrane or to secretion (Youn et al. 2010). The exact mechanism of NS1 hexamer formation is not clear, and three possible locations have been proposed including immediately after dimerization at the ER, along the Golgi secretory pathway, or it could occur when three dimers associate on the membrane surface causing disruption of the membrane and dragging a lipid cargo out in the process (Gutsche et al. 2011; Muller and Young 2013).

*In vitro* studies have shown that secreted DENV NS1 binds to specific glycosaminoglycans on the surface of infected and uninfected cells through its glycans expose on the protein (Avirutnan et al. 2007) and have been also reported that NS1 is internalized by endocytosis, where it accumulates in the late endosomes for up to 48 h (Alcon-lepoder et al. 2005) and cause hyperpermeability by destroying the endothelial glycocalyx (Puerta-Guardo, Glasner, and Harris 2016; Wang et al. 2019).

#### 1.5.5. NS1 circulation in the bloodstream of patients

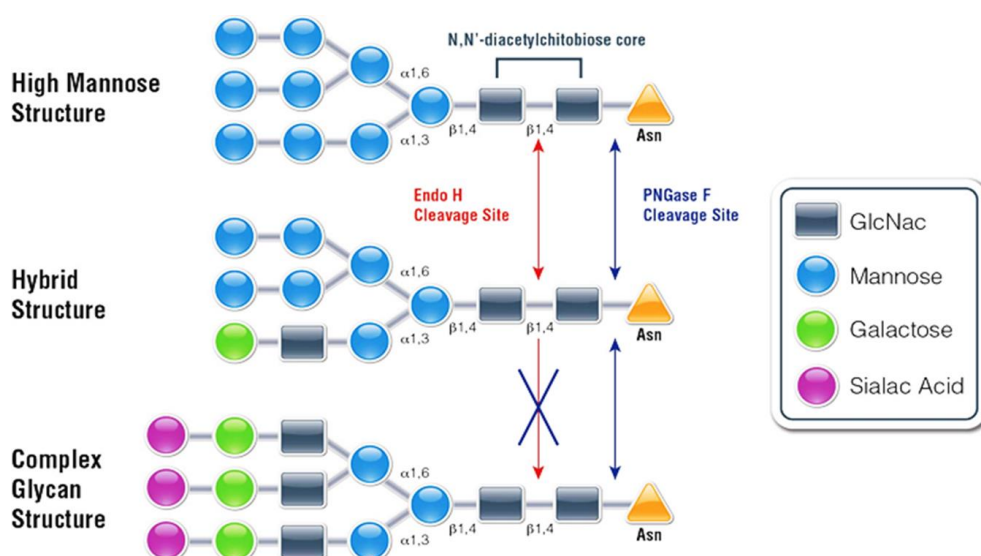
The presence of NS1 in the serum of infected patients have been reported for all flavivirus. However, the concentration of circulating NS1 varies depending on the individual, infecting virus, the course of infection as well as, the methodology used for the quantification. In a study performed to quantify the levels of NS1 viral antigen in plasma from infected patients with different DENV serotypes was found that the amounts of protein for DENV serotype 3 in infected patient is substantially lower (93ng/mL) compared to serotype 1 (285ng/mL) and serotype 2 (700ng/ml) (Alcon et al. 2002). While in another study were found lower and varied levels of NS1, from 22.6ng/ml to 36.8ng/mL in samples collected 2 and 7 days after onset of symptoms, respectively. Differences were not observed between primary and secondary DENV infections (Allonso et al. 2014).

Independently of the levels of NS1 in blood circulation, it has been demonstrated that secreted NS1 during infection with DENV is associated with disease severity (Avirutnan et al. 2006;

Libraty et al. 2002). Importantly, inoculation of mice with purified DENV 1-4 NS1 is able to induce vascular leakage and production of key inflammatory cytokines. *In vitro* and *in vivo* experiments have also shown that NS1 causes virus/tissue-specific endothelial hyperpermeability (Katzelnick et al. 2017; Killingbeck et al. 2015; Puerta-Guardo, Glasner, and Harris 2016; Wang et al. 2019). Together, these reports suggest the potential of NS1 as a candidate for flavivirus therapeutics.

### 1.5.6. Glycosylation of NS1 protein

Flavivirus NS1 proteins are characterized by two or three N-glycosylation sites. N-glycosylation involves the attachment of glycans on NS1 from a dolichol-phosphate-linked precursor oligosaccharide (usually is synthesized across the membrane of the ER) to the appropriate asparagine residues within the consensus sequence (Asn-X-Thr/Ser except where X is a proline) as the incipient protein is being translated. These N-linked glycans share a standard chitobiose core structure (including the first two N-acetylglucosamine residues and the first three mannose residues), and they can fall under three different structures (Figure 1.8): 1) high mannose glycans that contain unsubstituted terminal mannose sugars; 2) complex glycans that share a standard core structure but the high mannose glycans are processed to more complex sugars during the pass by the ER-Golgi pathway, and 3) hybrid glycan structures that are composed by a mix of high mannose and complex sugars (Aebi et al. 2009; Higuel et al. 2016).



**Figure 1.8. Representation of N-glycan structures present on NS1 protein.** Structures of high mannose, hybrid and complex glycans structures, as well as, the cleavage site of PNGase F (indicated with blue arrows) and Endo Hf (indicated with red arrows) endoglycosidases are indicated. The X symbol showed in blue indicates the inability of Endo Hf to digest complex glycan structures (adapted figure from Promega).

The glycosylation profile of NS1 protein varies depending on the infecting flavivirus, the host cell they infect, as well as, their cellular locations. Two conserved glycosylation sites are perfectly conserved among all mosquito-borne flaviviruses at positions N-130 and N-207 (208 for YFV virus, respectively) (Smith and Wright, 1985; Flamand, Deubel, and Glardt 1992; Pryor and Wright 1994; Yap et al. 2017; Zhao et al. 1987). While, a third glycosylation site at N-175 is present in all members of the Japanese encephalitis subgroup (west nile, japanese, murray valley, usutu, st. louis and tick-borne encephalitis viruses with the exception of JEV) (Bakonyi et al. 2004; Blitvich et al. 2001; Dalgarno et al. 1986; Coia et al. 1988). Notably, from this subgroup, the TBEV virus NS1 protein of both the Western and the Far Eastern subtype shares only the potential N-glycosylation site at amino acid 207 with other flaviviruses. In addition, the NS1 protein of TBEV contains two potential N-glycosylation sites at positions 85 and 223, which are not related to those of mosquito-borne flaviviruses (Mandl, Heinz, and Kunz 2004).

#### **1.5.6.1. Glycosylation pathway of NS1 protein**

The glycosylation of NS1 begins immediately after translation and cleavage into the lumen of the ER, when the monomer is altered at multiple sites by the addition of high-mannose carbohydrates. This is mediated by a host cell glycosyltransferase enzyme that recognizes asparagine residues within the consensus sequence (Gavel and Heyne 1990).

N-glycans display is different depending on the cell and NS1 location as they undergo modifications during the maturation process to achieve the final structure. In mammalian cells, the initial glycan structure is trimmed in the ER (Herbert, Foellmer, and Helenius 1995). Subsequently, during passage through the Golgi compartment, the high mannose carbohydrates are processed to hybrid or complex-type N-glycans by glycosyltransferases and glycosidases, respectively (Flamand et al. 1999; Mason 1989; Pryor and Wright 1994). On the contrary, the glycosylation profile of NS1 proteins produced in insect cells has been in controversy due to their incapacity to produce mammalian-like glycans (Rendi, Wilson, and Paschinger 2008; Yap et al. 2017). They are mainly constituted by high mannose structures. It has been demonstrated in several cell types, including *Aedes Aegypti* and *Aedes albopictus* insect cells (Hacker, White, and Silva 2009; Rhomberg et al. 2006).

Although both mosquitoes and mammalian cells can glycosylate NS1 proteins, only hexameric NS1 produced in mammalian cells can be Endoglycosidase Hf resistant (Endo Hf is unable to digest complex glycans). In These cells multi-branched complex type sugars are added to the

N130 glycan indicating the passage of secreted NS1 through the Golgi compartment (Mason 1989; Pryor and Wright 1994; Winkler et al. 1989), whereas the N207-glycans are retained in the high mannose form due to the inaccessibility for modification by Golgi-resident enzymes after the dimerization of NS1 (Flamand et al. 1999; Mason 1989; Pryor and Wright 1994).

The differences in the glycosylation profile of NS1 support some studies where have been reported that NS1 is secreted into the extracellular milieu from flavivirus-infected mammalian but not insect cells (Flamand et al. 1999; Muller and Young 2013). Nevertheless, the secretion of NS1 from insect cells is controversial since there are several reports showing NS1 detection in the culture supernatants of infected insect cells (Alcalá et al. 2016; Alcalá, Palomares, and Ludert 2018; Leblois and Young 1995; Ludert et al. 2008; Thiemmecca et al. 2016).

#### **1.5.6.2. Biological importance of NS1 glycosylation**

Proper glycosylation status of NS1 has been widely recognized to be essential for virus replication, dimer stability, and secretion from infected cells (Rastogi, Sharma, and Singh 2016; Somnuk et al. 2011). In Vero cells infected with DENV-1 and treated with inhibitors that block trimming of N-glycans in the ER or Golgi, it has been shown that glycosylation inhibition represses the secretion of soluble NS1 and it could be due to reduced stability and intracellular NS1 degradation (Flamand et al. 1999). Additional pioneers studies also showed that the inhibition of N-linked glycosylation with tunicamycin impairs NS1 processing and secretion in infected cells (Blitvich et al. 1999; Pryor and Wright 1994; Winkler et al. 1989).

Nevertheless, since tunicamycin might also inhibit glycosylation of cellular proteins, which may be involved in NS1 transport, the functional roles of N-linked glycosylation sites of NS1 have been determined in more recent studies by site-directed mutagenesis. In the case of DENV-2 NS1, the absence of the N-207 glycosylation site affects the secretion, stability of hexameric form, and cell surface expression of NS1 (Somnuk et al., 2011). However, a more recent study published in 2019 reported the purification of DENV2, WNV, and ZIKV NS1 containing a mutation at position N-207 from the supernatant of HEK-293F mammalian cells. The same studied showed that the mutation at position N-130 affected the secretion of NS1 from the same cells (Wang et al. 2019).

In the virus cycle, the glycosylation of NS1 is also essential for viral processes ranging from replication to virulence. The mutation of the N-130 glycosylation site of DENV-1 NS1 affects

the production of viral particles in mammalian and mosquito cells, and this effect can be rescued by exogenously expressing wild-type NS1 (Crabtree, Kinney, and Miller 2005; Tajima, Takasaki, and Kurane 2008). In human SW-13 infected cells with YFV, NS1 mutants lacking either one or both glycosylation sites (N-130 and N-208) produced small plaques, delayed cytopathic effect, low virus yields, impaired secretion, and deficient RNA accumulation. In addition, the elimination of the first glycosylation site led to a significant reduction in mouse neurovirulence (Muylaert et al. 1996).

The attenuation of infection has also been demonstrated in mice infected with WNV and DENV2. In both cases, the removal of the N-130 glycosylation site in NS1 reduced virus growth in cell culture (mosquito and mammalian cells) and decreased neurovirulence in mice (Crabtree, Kinney, and Miller 2005; Lin et al. 2015; Somnuk et al. 2011; Whiteman et al. 2011). On the contrary, the removal of the N-207 glycan in DENV 1 and 2 does not affect growth and virus titers in mammalian cells (Crabtree, Kinney, and Miller 2005; Tajima, Takasaki, and Kurane 2008). These findings have suggested that at least one of the N-glycosylation sites (probably N-130) is essential to produce viable virus in mammalian cells. This is in line with the most recent publication indicating that mutation of the N-130 glycosylation site affects the secretion of NS1 (Wang et al. 2019).

#### **1.5.7. Role of NS1**

Flavivirus NS1 plays a diversity of functions, including roles in viral replication, active participation in the pathogenesis, as well as, in the generation of protection. Intracellular NS1 is essential for viral RNA replication, as demonstrated by the colocalization of NS1 with the viral double-stranded RNA in vesicle packets and by leading dramatic defects in virus production when N-linked glycosylation sites are mutated (Muylaert et al. 1996; Rastogi, Sharma, and Singh 2016). Meanwhile, the secreted and membrane-associated NS1 have been shown to activate human complement (Conde et al. 2017).

An interaction between NS1 and the host immune system has been known since NS1 was first identified as a soluble complement-fixing antigen (Brandt, Cardiff, and Russell 1970). NS1 has the ability to activate but also to inhibit the host immune system. In dengue virus infection has been shown that high levels of soluble and membrane-associated NS1 activate human complement, establishing a positive correlation with disease severity. It probably occurs at the sites of vascular leakage in patients with dengue hemorrhagic fever (Avirutnan et al. 2007; Sun

et al. 2007). The soluble NS1 is also able to induce pro-inflammatory cytokine response and vascular leakage via the Toll-like receptors expressed on endothelial and immune cells (Modhiran et al. 2015).

In addition to being involved in the activation of the complement system, soluble flavivirus NS1 has a direct immune evasion function antagonizing complement activation (Muller and Young 2013). DENV secreted NS1 was identified to bind to the complement protein C4 promoting its cleavage to C4a and C4b (Avirutnan et al. 2010). This mechanism limits the amount of C4 available and, therefore, can protect the virus from neutralization. Furthermore, DENV-1 NS1 protein is endocytosed by human Huh7 and HepG2 hepatocytes *in vitro* and is accumulated in the late endosomal compartment suggesting that the secreted NS1 possibility contributes to viral propagation *in vivo* (Alcon-lepoder et al. 2005). Endocytic Internalization of DENV, WNV, and ZIKV NS1 proteins and induction of tissue-specific endothelial hyperpermeability has been confirmed in *In vitro* and *In vivo* experiments (Puerta-Guardo et al. 2016; Wang et al. 2019).

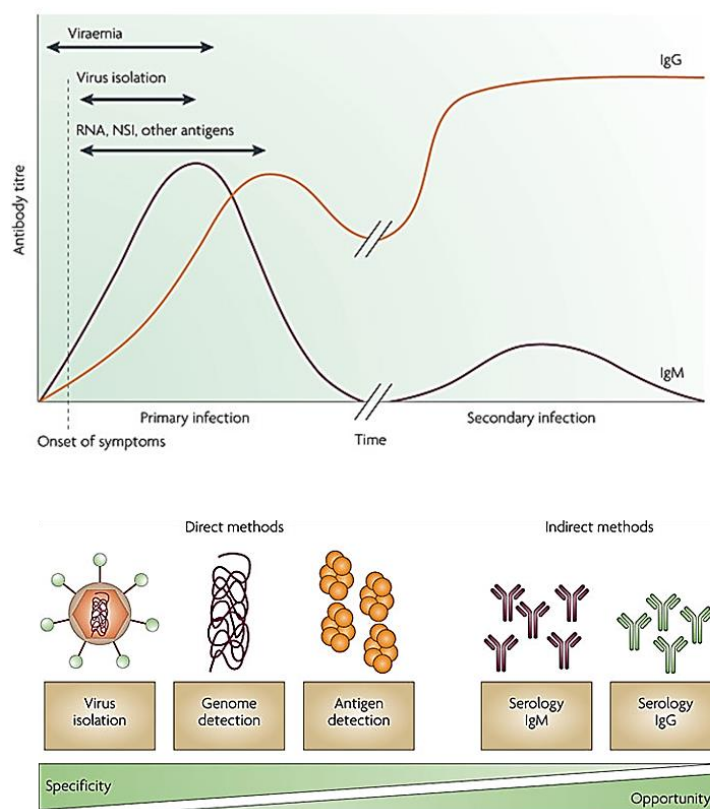
Flavivirus infection elicits a high concentration of antibodies to NS1 in circulation. Therefore, much effort has been made to develop diagnostic tests to detect the presence of NS1 protein and antibodies in serum from infected patients. Various attempts have also been made for NS1 based subunit and DNA vaccine against several flaviviruses. However, safety and efficacy are still a major challenge for most of the flavivirus infectious.

### **1.6. The immune response against flavivirus**

Mammalian cells have evolved an innate and adapted immune response to detect, contain, and clear the viral infections. The innate immune response offers the first protection against pathogens and is mediated by germline-encoded pattern recognition receptors (PRRs) that sense viral RNA, as retinoic acid-inducible gene I (RIG-I), Toll-like receptors 3 and 7 (TLR3 and TLR7) and melanoma differentiation associated gene 5 (MDA5). While adapted immunity is implicated in pathogens clearance during the late phase of infection and involves lymphocytes T and B expressing antigen-specific receptors (Diamond 2003).

A few days after the infection with flavivirus, the production of antibodies is primarily directed against the virus envelope proteins because they are the major component of the viral particle. Although NS1 is absent from the virion, antibodies against soluble NS1 secreted by infected cells have been detected in both human patients and murine models infected with flaviviruses

(Rey et al. 2018). In the case of DENV infections, the immune response varies depending on whether it is a primary or secondary infection. Secondary infection by a different DENV serotype is considered the most significant risk factor in developing dengue hemorrhagic fever and dengue shock syndrome. It is due to the presence of circulating non-neutralizing, cross-reactive antibodies that increase the virus entry into the target cell through the cell Fc receptor (Bruhns, 2012).



**Figure 1.9. Representation of methods and biomarkers for flavivirus diagnosis.** In the upper panel is represented the antibody response against flaviviruses during primary and secondary infections. In the lower panel is showed some direct and indirect methods used in flavivirus diagnosis, indicating the specificity and opportunity in terms of availability.

As shown in figure 1.9, primary infection is characterized by a slow and low-titer IgM antibody response that can be detected in most patients by day 3-5 of illness. The IgM peaks approximately two weeks after the onset of symptoms and remains high for up to three months following the infection. IgG antibodies are detected at low titer a couple of days after the IgM and increase slowly. IgG antibodies remain stable for decades and confer protection (Vaughn et al. 2000). For this reason, a test performed to detect the presence of IgG antibodies in an acute phase patient can turn out positive, to indicate past exposure to a related flavivirus.

## 1.7. Diagnosis of flavivirus

The laboratory diagnosis of flavivirus infections can be performed through a variety of assays that can be divided into direct and indirect methods (Figure 1.9, lower panel). Virus isolation and detection of the genome/antigens are direct techniques that can be used during the viremic phase. These techniques are highly specific, but they are expensive, require a skilled operator, and detect the virus only during the short-lived viremic phase. In contrast, indirect methods are mainly based on serological assays to measure the presence of IgM and IgG antibodies. Many of these methods suffer from low specificity because of the broad antigenic cross-reactivity that exists between all flaviviruses. Nevertheless, they are the most widely used approaches, especially in resource-limited settings (Mardekian and Roberts, 2015).

The development of specific and sensitive assays is one of the main priorities for the diagnosis of flavivirus infections. It can be done by golden standard techniques such as the plaque reduction neutralization tests (PRNT) or by more advanced technologies in point-of-care (POC) formats that pretend to provide a rapid, low-cost, and precise diagnosis. It is challenging depending mainly on what stage in the infection the analysis is performed, the immune status of the patient because of natural infection or vaccination, the diagnostic test applied, the geographic region where the patient lives, clinical presentation, and many other factors that can complicate the disease spectrum (Ceianu, Cotar, and Bădescu 2018; Bhat et al. 2015).

### 1.7.1. Virus isolation

Virus isolation is considered the traditional method for flavivirus diagnosis. The virus may be isolated during the viremic phase (figure 1.9 upper panel, first days of illness) from serum, plasma, leukocytes, whole blood, urines, cerebrospinal fluid, as well as from tissues obtained at autopsy. Importantly, the specimen has to be properly transported and stored to preserve the viability of the virus (Fatima and Wang 2011; Scott and Nisalak 1980).

Basically, the clinical samples taken from patients are cultured in a variety of cell lines that represent epithelial, endothelial and glial cells, from lung, kidney and brain tissues, derived from insect (C6/36, *Aedes albopictus* and AP-61, *Aedes pseudoscutellaris*), humans (HBMEC, A549, U87, and HeLa), non-human primates (Vero, LLCM-K2), and rodents (BHK-21, C6) (Samuel and Tyagi 2006; Coelho et al. 2017). After an incubation period permitting virus replication, viral identification can be performed using specific monoclonal antibodies in



immunofluorescence assays or by molecular techniques. Although virus isolation is a reference method in flavivirus diagnosis, it is not particularly practical in clinical diagnosis management, as isolation can take days to weeks to perform the test, and because of high costs that procrastinate the inevitable vector control under epidemic situations.

### **1.7.2. Plaque reduction neutralization test**

Plaque reduction neutralization test (PRNT) is a method for measuring specific antibodies that neutralize and prevent viral infection in cultured cells. The results are measured by microscopic observations, fluorescent antibodies, or specific dyes that react with the infected cell. The PRNT has been considered the most virus-specific serological test among the flaviviruses, and frequently, it is used in infection with dengue virus for differentiating viral serotypes following a primary infection (Roehrig, Hombach, and Barrett 2008).

Although the PRNT typically provides the highest specificity, partial neutralization due to cross-reactive antibodies has also been reported. Current reports have suggested the depletion of IgG antibodies to increase the specificity of the assay (Calvert et al. 2018; Singh et al. 2018). In addition, the PRNT is a labor-intensive technique requiring at least one week to get results and not readily amenable to high throughput, making it difficult to use for large-scale surveillance and vaccine trials (Roehrig, Hombach, and Barrett 2008; Thomas et al. 2010).

### **1.7.3. Molecular assays**

Standard laboratory tests for specific diagnosis of flavivirus infection are based on the molecular detection of the viral RNA genome. The most appropriate period to perform these assays is within a week from the onset of symptoms when high levels of viral particles rich the viremic phase of the infection (Mazeaud et al. 2018). A positive result on the detection of the viral RNA is a marker of a current viral infection. Meanwhile, a negative result could be an indication of the absence of the virus, but also, it could be because the test was done either too early or after the viremic phase. Therefore, if the clinical presentation of the patient suggests the viral infection, the results should be confirmed by serology and, eventually, by the plaque reduction neutralization test (PNRT) (Charrel et al. 2016; Samuel and Tyagi 2006).

Molecular methods such as Reverse-Transcription PCR (RT-PCR) or Real-Time PCR (qPCR) are direct techniques that are commonly used during the viremic phase when the virus genome is present in peripheral blood (Sekaran and Artsob 2007). These methods provide same-day or

next-day diagnosis during the acute phase of the disease. In the case of dengue virus detection, there are several single-step multiplex RT-PCR assays that have been developed for DENV serotyping (Alm et al. 2015; Chang and Vorndamt 1992; Mun et al. 2019). Similar techniques have been developed for molecular diagnosis of all flaviviruses. Importantly, If the assay is performed during the viremic phase, molecular techniques allow the detection of the viral RNA with high sensitivity, specificity, and faster compared to other assays such as virus isolation methods (Deubel et al. 1990). However, they are more expensive and require trained personnel and equipment (Peeling et al., 2010; Muller, Depelsenaire, and Young, 2017).

Innovative and new technologies based on molecular detection of flavivirus infections have been developed. They range from classical assays using a thermocycler for nucleic acid amplification to thermocycler-free technologies and point of care tests (Chen et al. 2019; Goncalves et al. 2018; Mauk et al. 2017; Sekaran and Artsob 2007). In the case of thermocycler-free technologies such as the loop-mediated isothermal amplification of viral RNA (LAMP), they mimic *in vitro* nucleic acid amplification showing high levels of sensitivity and specificity when used alongside other diagnostic methods. As an example, a LAMP for detection of ZIKV ARN has shown excellent performance in RNA detection from mosquitos, and humans samples (Chotiwan et al. 2017; Mora-Cárdenas and Marcello 2017).

#### **1.7.4. Serological assays**

Serological assays are the most widely applied approaches in routine diagnosis as they are relatively inexpensive and easy to perform compared with culture and nucleic acid-based methods. Serology is dominated by low-cost and easy to use mono-parametric strip tests, ELISA assays, indirect immunofluorescent methods, hemagglutination inhibition, and neutralization techniques. However, the main limitation of serological methods is the broad antigenic cross-reactivity of anti-flavivirus antibodies, prior exposure to related flaviviruses, or vaccines (Hunsperger et al. 2014; Lee et al. 2019; Muller, Depelsenaire, and Young 2017; Sekaran and Artsob 2007).

Moreover, the overlapping geographic distribution of flaviviruses and international travels increases the problem of cross-reactivity, making difficult the diagnosis but also the surveillance of flaviviruses. There is a growing demand for syndrome-based multi-parametric devices with an electronic interface to process and archive the data (Marcello et al., 2013; Tagliabue et al., 2017). However, specific assays for differential flavivirus diagnosis are still in

development. They are urgently needed, especially in those countries where more than one flavivirus is circulating.

#### **1.7.4.1. Detection of IgM and IgG antibodies by ELISA**

The detection of IgM and IgG antibodies by ELISA is one of the most potent serological diagnostic methods for routine flavivirus infection. Monitoring antibodies in sera samples are highly useful to identify the virus and the stage of the infection. The presence of IgM antibodies is indicative of recent exposure to the virus, which is helpful in the diagnosis of symptomatic or asymptomatic patients who are RT-PCR negative. While the detection of IgG antibodies could be an indication of either a past infection when the test is positive during the acute phase, or it is a current infection if the test is positive at late stages of the acute phase and during the convalescent phase of the infection (Gubler et al. 2017; Muller, Depelsenaire, and Young 2017).

There are several commercial assays for direct and indirect detection of IgM and IgG antibodies. A summary of the most commonly used commercial assays in laboratory diagnosis is shown in table 1.1. IgM/IgG capture ELISA (MAC/GAG-ELISA) assays allow the detection of IgM/IgG antibodies present in the serum by first capturing all IgM or IgG antibodies through anti-human-specific IgM/IgG coated in the solid phase of ELISA plates. Then, the captured antibodies react with flavivirus specific antigens. There are also commercially available assays for direct detection of IgM and IgG antibodies. They are based on the direct detection of antibodies against the inactivated virus, structural proteins (mostly envelope protein), and a few numbers of assays have implemented the use of NS1 protein as an antigen. Independently whether the antibody detection is made by direct or indirect methods, many of them detect high cross-reactive antibodies against structural proteins. In all cases, the comparison of different commercial assays has demonstrated many differences in terms of sensitivity and specificity between the results when the same samples are analyzed with different assays. It has been shown for TBEV (Ackermann-gäumann et al. 2018; Velay et al. 2018), WNV (Hukkanen et al. 2006; Malan et al. 2004; Niedrig et al. 2007), ZIKV: (Basile et al. 2018; Kikuti et al. 2018; L’Huillier et al. 2017; De Ory et al. 2018; Safronetz et al. 2017), USUV: (Gaibani et al. 2012; Saiz and Blázquez, 2017), and DENV: (Aryati et al. 2013; Chien et al. 2018; Hunsperger et al. 2014; Lee et al. 2019).

**Table 1.1. Commercial ELISA assays commonly used for the diagnosis of flavivirus infection.**

VIRUS	DETECTION	COATING	ELISA/COMPANY
TBEV	Direct IgM and IgG detection	purified preparations of tissue cultured virus as the antigen	ELISA-VIDITEST VIDIA, Jesenice, Czech Republic
			TBE virus EI 2661-9601, Euroimmun, Lübeck, Germany
			EC 117-00/FSME/TBE ELISA, Sekisui Virotech, Rüsselsheim, Germany
			Enzygnost Anti-TBE/FSME/ETG Virus, Siemens, Marburg, Germany
			RIDASCREEN FSME/TBE Virus RIBIOPHARM, Darmstadt, Germany
			SERION ELISA classic TBE Virus Virion/Serion, Würzburg, Germany
WNV	IgM capture /direct IgG detection	anti-human IgM antibodies/recombinant WNV antigen	Focus Diagnostics ELISA, Cypress, CA
		anti-human IgM antibodies/Flavivirus antigen (DENV1-4)	PanBio ELISA, Columbia, MD
	Direct IgM and IgG detection	Cell derived whole virus antigens/WNV recombinant antigen	EUROIMMUN, Luebeck, Germany
	IgM Capture/Direct IgG detection	anti-human IgM antibodies/WNV-derived recombinant antigen	InBios ELISA, Seattle, WA
ZIKV	Direct IgM and IgG detection	ZIKV NS1 recombinant antigen	EUROIMMUN, Luebeck, Germany
	IgM capture ELISA	recombinant ZIKV envelope glycoprotein	InBios International Inc., Seattle, WA
		unspecified Zika virus antigen	NovaTec Immundiagnostica GmbH, Dietzenbach, Germany
			Abcam, Cambridge, UK
USUV	Direct IgG detection	Recombinant ZIKV envelope protein	EUROIMMUN, Luebeck, Germany
DENV	IgM and IgG capture ELISA	anti-human IgM/IgG	Focus Diagnostics ELISA, Cypress, CA
			Standard Diagnostics Inc., South Korea
	Direct IgM and IgG detection	Recombinant envelope antigen	Panbio Diagnostics, Alere, Australia
	IgM capture ELISA and direct IgG detection	anti-human IgM/recombinant antigen	InBios International, Inc.
	Direct IgM and IgG detection	Recombinant envelope antigen	Abcam, Cambridge, UK
			EUROIMMUN, Luebeck, Germany

### 1.7.5. Antigen detection

Envelope and the NS1 proteins are the main immunogenic targets of the antibody response during flavivirus infections. Envelope protein is the major virion surface protein and plays an important role during the entry of the virus into the host cell (Butrapet et al. 2011; Muller and Young 2013; Kaufmann and Rossmann, 2011). The envelope protein is composed of three different structural domains named I, II, and III that are discontinuous when compared against the primary structure of the protein (Kuhn et al. 2002; Modis et al. 2004).

Based on the sequencing of *in vitro* neutralization escape variants, many neutralizing antibodies against flaviviruses localize to the DIII domain (Beasley and Barrett 2002; Volk et al. 2004). Coincidentally, this is also the most variable domain between DENV serotypes, which means that these antibodies are usually highly specific and explain why DIII has been considered for vaccine development (Crill and Roehrig 2001; Pierson and Diamond 2008;

Wahala et al. 2009). However, studies have shown that after natural dengue infection, the immune response is dominated by highly cross-reactive, weakly neutralizing antibodies, directed mostly against an epitope on DI/DII (de Alwis et al. 2011). Specifically, many of these antibodies are directed to the fusion loop domain that is highly conserved between flaviviruses (Zhang et al. 2017). For this reason, some groups have developed ELISA assays based on envelope protein containing mutations in the fusion loop. However, the problem remains unsolved (Rockstroh et al. 2015, 2017).

The development of specific diagnostic assays for the differential diagnosis of flavivirus infections is focused on NS1 protein. As mentioned above, the NS1 glycoprotein can be found attached to the surface of infected cells and is also secreted as a hexamer. This explains why, despite being a non-structural protein, it is among the most significant targets of the antibody response (Muller and Young 2013). When compared with antibodies induced against structural proteins, including the envelope protein, antibodies against NS1 show restricted cross-reactivity between several flaviviruses (Cleton et al. 2015; Dejnirattisai, 2010).

#### **1.7.5.1. NS1 as a diagnostic biomarker**

NS1 protein is one of the major viral immunogens upon flavivirus infection and for this reason, several groups have developed capture enzyme-linked immunosorbent assays (ELISA) to understand the biological relevance of NS1 secretion and its potential use as a diagnostic marker (Balmaseda et al. 2017; Bosch et al. 2017; Kathiresan et al. 2017; Lebani et al. 2017; Lemes et al. 2005; Röltgen et al. 2018; Rose et al. 2016).

Detection of NS1 proteins from different flaviviruses by antigen-capture ELISA has been demonstrated from the first days after the onset of symptoms until almost 2 weeks when the clinical manifestation of the disease is over in most of the cases. The levels of NS1 varies, ranging from 1ng/mL and at high levels with an accumulation of up to 50 µg/mL in the serum of infected patients but not in convalescent sera recovered after day 15 (Alcon et al. 2002; Macdonald et al. 2005). This data is correlated with the detection of the viral RNA by RT-PCR, including NS1 detection several days after viremia had become undetectable or in the presence of IgM antibodies. It suggests that NS1 is present at a higher magnitude than virus particles release and converts it an important marker for antigen/antibody detection in infected patients (Alcon et al. 2002). The window of time for NS1 detection and antibodies is important, especially for some flaviviruses such as WNV, where the amplitude and duration of viremia are

relatively low and short compared with other flaviviruses (Busch et al. 2005; Vaughn et al. 2000).

The capture of NS1 protein through monoclonal antibodies is a potential biomarker to optimize the specificity of immune assay tests. The application of monoclonal antibodies provides the ability to bind specifically and with high-affinity NS1 protein from different flavivirus (Balmaseda et al. 2017; Rose et al. 2016). The generation of these antibodies is mainly based on the immunization of animals using recombinantly expressed on the cell surface or purified NS1 proteins (Röltgen et al. 2018). However, the identification of specific NS1-reactive monoclonal antibodies is challenging. It has stimulated many groups to combine capture of NS1 with IgM/IgG antibody detection to improve the sensitivity and specificity in flavivirus diagnosis (Fry et al. 2011).

#### **1.7.5.2. IgM and IgG detection based on NS1 protein**

As was mentioned above, NS1 protein is one of the major targets of the following infection with flavivirus. NS1 antigen detection has a limited time window, usually within the first few days after the onset of symptoms (Ahmed and Broor 2014). In contrast, the detection of antibodies anti NS1 proteins has a broader detection window, from the first days (2-3 days) of infection until long term depending on the antibody. It indicates their potential role as biomarkers of past and recent flavivirus infections (Nascimento, George, et al. 2018; Nascimento, Huleatt, et al. 2018).

Evaluation of acute infection by measuring anti-NS1 IgM antibodies has shown higher sensitivity compared to RT-PCR in sera samples of infected patients with dengue virus. Already on days 4–5, IgM was detected, while on days 6–7, NS1 antigen and IgM detection were superior to RNA detection, and this was also the case for the samples taken on later time-points (Huhtamo et al. 2010; Sankar et al. 2013). High sensitivity and specificity have also been demonstrated for anti NS1 IgG antibodies. The potential of flavivirus NS1 protein for surveillance and specific diagnosis of multiple flavivirus infections has already been demonstrated by protein microarray-based on flavivirus NS1 proteins obtained commercially (Cleton et al. 2015). These findings make antibody detection against NS1 protein a powerful approach for adequate differential diagnosis between flavivirus. However, there is only one NS1-based ELISA commercially available (Euroimmune) for ZIKV diagnosis.

## 1.8. AIM OF THE STUDY

The main aim of this Ph.D. thesis was to develop a multiparametric ELISA for a specific diagnosis of flavivirus infection (TBEV, WNV, ZIKV, USUV and DENV1-4) through the detection of IgM and IgG antibodies against NS1 proteins. It involved the following tasks:

- **Cloning, expression, and purification of native recombinant NS1 proteins**
  - Cloning and expression of Histidine-tagged NS1 genes
  - Optimization of strategies to express and purify NS1 proteins
  - Purification of NS1 proteins in large amounts by affinity chromatography
  - Analysis of the glycosylation and oligomeric status of purified NS1 proteins
- **Validation of the immunological properties of purified recombinant NS1 proteins**
  - Cloning and expression of V5-tagged NS1 genes
  - Mice immunization with plasmids encoding V5-tagged NS1 genes
  - IgM/IgG detection from sera of immunized mice
  - IgM/IgG detection from sera samples of RT-PCR confirmed infected patients with TBEV, WNV, ZIKV, USUV, and all four DENV serotypes
- **Analysis of the native conformation of NS1 proteins for antibody detection**
- **Molecular detection and seroprevalence studies:**
  - WNV and USUV in blood donors from North-Eastern Italy
  - Flaviviruses in febrile-illness patients from Borno-State, North-Eastern Nigeria

# MATERIALS & METHODS

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## 2. MATERIALS AND METHODS

### 2.1. Cell culture

Human embryonic kidney 293T cells (HEK-293T, source ATCC CRL-7216) were cultured in commercial Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) containing GlutaMAX™ supplement, High Glucose (4.5g/L) and sodium pyruvate. Culture media was supplemented with 10% fetal bovine serum (FBS) (Life Technologies), and monolayers of HEK-293T cells were grown at 37°C in 5% CO<sub>2</sub>. The cells were passaged every 3-4 days with a seeding density of  $5.0 \times 10^6$  cells in 150 mm dishes to keep them in culture.

### 2.2. Plasmid construction

The nucleotide sequences of different representative flaviviruses strains coding for NS1 protein were derived from the NCBI database as follows:

- TBEV Neudoerfl strain (NC\_001672.1),
- WNV New York 1999 (NY99) strain (NC\_009942.1),
- ZIKV São Paulo 2015 (SPH2015) strain (KU321639.1),
- USUV Vienna 2001 strain (AY453411),
- DENV1 Hawaii A strain (NC\_001477.1),
- DENV2 New Guinea C (NGC) strain (NC\_001474.2),
- DENV3 Sri Lanka strain (NC\_001475.2), and
- DENV4 Dominica strain (TC25) strain (NC\_002640.1).

Sequences were codon-optimized for the expression in *Mus musculus* and were synthesized commercially by *Gene Art Gene Synthesis* (Thermo Fisher Scientific). The genes were kept in-frame with the polyhistidine (6x-His) tag or the V5 tag (GKIPNPLLGLD) at the C-terminus. Only for WNV-NS1 two-point mutations were inserted (RQ10NK), which have been shown to increase NS1 secretion (Youn et al. 2010). Codon-optimized sequences were purchased as synthetic genes in pMA-T vectors (Thermo Fisher Scientific) and sub-cloned into the pcDNA3.1 expression vector (Life Technologies) containing an immunoglobulin leader sequence (Sec) at the N-terminus. BssHII and ApaI restriction endonucleases (New England Biolabs, NEB) were used to digest the pMA-T and pcDNA3.1 vector. Ligation of the insert (NS1 gene) with the vector (pcDNA3.1) was performed, and MAX efficiency DH10β competent cells were used for

transformation. Colonies were selected based on expected BssHII and Apal double digestion profiles and correct sequencing results.

To generate a pcDNA3.1 construct containing the TBEV NS1 coding gene fused to a V5 tag upstream of the histidine tag at the C-terminus, I designed a set of oligos (forward and reverse) containing both tags between KpnI and Apal restriction sites at the N-terminus and C-terminus, respectively. pcDNA3.1 vector containing the TBEV NS1 gene was double digested with KpnI and Apal to remove the 6xHis tag at the C-terminus, and then, the vector was used to clone the V5-6xHis oligos. Cells were transformed, and colonies were selected, as it was mentioned previously.

### **2.3. Expression of recombinant NS1 proteins**

Transient transfection of HEK293T cells was performed by the standard calcium phosphate method (Sambrook, 1989). For small scale transfection, cells were seeded in 6-well plates at  $0.5 \times 10^6$  cells/well density 24h before transfection. Independently of the plasmid, freshly replated low-passage cells were overlaid with the calcium-phosphate-DNA suspension prepared by mixing 2.5µg plasmid DNA with 2.5M NaCl and 2X Hepes buffered saline buffer (HBS pH 7.1). 16h after transfection, cells were washed twice in phosphate-buffered saline (PBS), and further cultured for 30h in serum-free media supplemented with 5 mM of sodium butyrate. Culture supernatants were then cleared by centrifugation at 4000 g for 4 min, while total cellular extracts were lysed in TNN buffer (100 mM Tris-HCl, pH 8, 250 mM NaCl, 1% NP-40) supplemented with Protease Inhibitor Cocktail (PIC, Sigma, P8340) at 4°C. Cellular extracts and supernatants were kept at -20°C until use. Expression and secretion of NS1 proteins were screened by western blot using specific monoclonal antibodies.

Large-scale transient transfection of HEK-293T cells was performed for NS1 protein purification. Cells were seeded in 150 mm plates at  $5.0 \times 10^6$  cells/well density 24h before transfection (20 plates were transfected in each purification). Independently of the plasmid, 20µg of DNA was transfected following the same protocol mentioned above. Culture supernatant containing secreted NS1 was collected 30h after transfection, cleared by centrifugation at 4000 g for 4 min, and used immediately for protein purification.

## 2.4. Western blot analysis

Samples were separated by Poly-Acrylamide Gel Electrophoresis (PAGE) under native, reducing, or non-reducing conditions, as indicated in each experiment. Native western blot was performed by removing sodium dodecyl sulfate (SDS) from the electrophoresis buffer (25 mM Tris, 190 mM glycine, 0.1% SDS), and from the Laemmli sample buffer (LB, 25mM Tris-HCl pH6.8, 1% SDS, 10% glycerol), as well as, the removal of 175 mM 2-Mercaptoethanol (2-βME), and without boiling the samples. Non-reducing western blot was done by separating the samples in the presence of LB without 2-βME. Samples were boiled for 12 minutes at 95°C and run in the presence of SDS when the western blot was performed under reducing or non-reducing conditions. In all cases, samples were centrifuged for 1 min at RT at 1000g before loading the gel.

Samples were run in electrophoresis buffer at 80 V into the stacking gel and later at 130 V into the running gel. After electrophoresis, proteins were transferred on nitrocellulose membrane (GE Healthcare - 10600015) and blocked with 5% milk solution in TBS (50 mM Tris-Cl, pH 7.6, 150 mM NaCl) plus 0.5% Tween-20 (TBST). When detecting V5-tagged proteins, membranes were incubated for 1h with an anti-V5 monoclonal antibody (1 µg/ml in TBST), washed, and probed with HRP-conjugated anti-mouse IgG goat antibodies (KPL, Gaithersburg, MA, USA, 074-1809) for 1h. In the case of His-tagged proteins, membranes were incubated for 1h with anti-his HRP-conjugated monoclonal antibody (Sigma-Aldrich). As a loading control, rabbit antibody anti-actin (Sigma) was used. Blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore – WBKLS0500) according to the manufacturer's instructions.

## 2.5. Purification of His-tagged NS1 proteins

Purification of recombinant NS1 (rNS1) proteins from clarified culture supernatants was performed by Fast Protein Liquid Chromatography (FPLC) using HiTrap Chelating HP 5mL columns (GE Healthcare) following the manufacturer's instructions. Briefly, the column was prepared by loading it with 8mL of 10mM Nickel, followed by washes with distilled water. Then, the column was equilibrated with binding buffer (0.02M sodium phosphate, 0.5M sodium chloride, pH 7.4) by washing with approximately 10-15 column volumes.

The supernatant containing 0.5M of sodium chloride (to improve the specificity of the binding) was loaded with a flow rate of 2.5mL/min. A wash with 5-10 column volumes with binding buffer was performed, followed by the elution of the His-tagged NS1 proteins using a linear gradient of Imidazole (100% in 10 min) present in the elution buffer (0.02M sodium phosphate, 0.5M sodium chloride, 0.5M imidazole, pH 7.4). All the collected fractions were analyzed by western blot using an antibody against the histidine tag. The positive fractions for the rNS1 protein were concentrated and buffer-exchanged to PBS using Ultra-4 centrifugal filters devices (Amicon 10K, Millipore - Germany). The purity and concentration of the purified NS1 proteins were estimated by coomassie blue staining and by Bradford assay, respectively. Densitometric analysis was also performed using Image Lab™ Software 6.0.1 (Bio-Rad).

## **2.6. Protein deglycosylation assay**

Purified NS1 proteins were digested for 1.5 hours with endoglycosidase Hf (Endo Hf) or/and Peptide-N-Glycosidase-F (PNGase F) endoglycosidases according to manufacturer's protocols (New England Biolabs). Briefly, 5µg of purified NS1 protein was denatured by heating reaction at 100°C for 10 minutes in the presence of denaturing buffer, followed by digestion with PNGase F and/or Endo Hf for 1.5h at 37°C. 200 ng of deglycosylated protein was loaded for western blot analysis. The denaturing step was not included when the NS1 proteins were used for NS1-based ELISA. In all cases, Reactants were subjected to 10% SDS-PAGE under reducing conditions followed by western blot using an anti-histidine monoclonal antibody.

## **2.7. Gene Gun-Mediated DNA Immunization**

Female *Balb/c* mice (5 to 6 weeks old) were immunized intradermally with a plasmid encoding for V5-tagged rNS1 protein. The V5 tag was used in the mice experiments to avoid cross-reactivity of the immunized sera towards the His tag. Instead of purifying each of the seven NS1 antigens for immunization I decided to go for the intradermal gene gun route.

Four mice were immunized for each viral NS1, and they were distinguished with an earmark (Rx, right; Lx, left; RxLx, right and left; and, unmarked). They were immunized by Gene Gun technology (Bio-Rad, Hercules, CA, USA) following a scheme based on prime-boost immunization strategy. Before the immunization, the abdominal area of each mouse was shaved, and 1 µm gold particles coated with 1 µg of plasmid DNA (V5-tagged construct of NS1 TBEV, WNV, ZIKV, USUV, and all DENV serotypes, 1-4) were delivered by biolistic particle

system at 400 psi. Each group of mice was immunized 4 times with a specific plasmid at fourteen days intervals. Blood samples were collected by sub-mandibular puncture before immunization (pre-immune sera) and 5-7 days after each boost (bleeding I, II, III, and IV). Sera samples were collected and stored at -20°C until use.

## **2.8. Identification of USUV**

The first screening of blood donors was routinely performed with the Cobas® WNV nucleic acid test (Roche). Confirmation of USUV RNA was obtained as described earlier (Cavrini et al. 2011). The primers (USU-F 5'-AAAAATGTACGCGGATGACACA-3', USU-R 5'-TTTGGCCTCGTTGTCAAGATC-3') amplified a partial sequence (73 bp) of USUV NS5 gene, which was detected by a dual-labeled probe (USU-P 5'- 6famCGGCTGGGACACCCGGATAACC-tamra-3').

Sequence analysis was performed on a PCR amplification product of 659bps from the NS5 gene using primers USU-9170F and USU-9704R (Weissenböck et al. 2004) and MAMD and cFD (Kuno et al. 1998). The evolutionary history was inferred using MEGA X software (Kumar et al. 2018; Saitou and Nei 1987). The phylogenetic tree was obtained by the Neighbor-Joining method on amino acid sequences; the Kimura two-parameter method was used to calculate nucleotide substitutions, and a bootstrap of 500 replicates lead to evaluate the significance of tree topology. Sequence data were directly submitted to GenBank with accession number: BankIt2264908 USUV/HU/FVG.ITA/2018/01, MN509808.

## **2.9. Human sera samples**

Sera samples from RT-PCR confirmed flavivirus infected patients were used for the validation of the purified NS1 proteins. These samples were well characterized by RT-PCR and commercial ELISA assays according to the manufacturer's instructions. The data of the characterization of these samples were provided by prof. Tatjana Avšič from the University of Lubiana.

### **2.9.1. Sera samples from RT-PCR confirmed flavivirus infected patients**

A total of 100 sera samples of TBEV and 16 samples of WNV patients were anonymously obtained from endemic areas in Slovenia and Italy, respectively. 15 for either ZIKV or DENV1, 2 or 3, and 8 for DENV4 sera samples were obtained from Slovenian travelers. The positivity of each serum sample was confirmed by RT-PCR and by commercial ELISA assays. A group of 43

sera samples, including TBEV/YFV, vaccinated people, and vaccine breakthrough patients, were also analyzed in this work.

The commercial ELISA assays used for the characterization of these samples include:

- TBEV, Enzygnost Anti-TBE/FSME ELISA (plates coated with inactivated TBEV).
- WNV, Focus Diagnostics ELISA (plates coated with recombinant envelope protein).
- ZIKV, Euroimmune NS1 ELISA (plates coated with recombinant ZIKV NS1 antigens).
- Independently of the DENV serotype, all DENV sera samples were tested using NovaTec ELISA (plates coated with envelope protein of DENV2).

### **2.9.2. Sera samples from blood donors North-Eastern Italy**

A total of 142 sera samples from blood donors from the Friuli-Venezia Giulia region were obtained in collaboration with Prof. Pierlanfranco D'Agaro from Burlo Garofalo Hospital in Trieste. Among them, 3 sera samples were obtained from an asymptomatic blood donor who was positive for WNV in the first screening by PCR at the regional transfusion blood center in Palmanova – Italy. The first collected sample from this patient was later confirmed for USUV by real-time RT-PCR amplification protocol of Cavrini et al. (Cavrini et al. 2011) and sequencing analysis. All 142 samples were analyzed for the detection of IgG antibodies against WNV by commercial ELISA (Focus Diagnostics). Since USUV is not considered an endemic virus in the region, only 44 samples were analyzed for the presence of IgG antibodies using the commercial ELISA (plates coated with USUV envelope protein) from Euroimmune.

### **2.9.3. Sera samples from febrile illness patients from Nigeria**

A total of 200 sera samples from febrile illness patients from North-Eastern Nigeria were also tested by NS1-based ELISA developed in this study. Incomplete clinical data was obtained from this set of samples.

## **2.10. Ethics statement**

Human sera were obtained from an established collection at the Institute of Microbiology and Immunology of the Faculty of Medicine at the University of Ljubljana, Trieste, and Maiduguri that were previously approved by the Medical Ethics Committee of their respective institutions.

Animal care and treatment were conducted in conformity with institutional guidelines after approval by the ICGEB Institutional Review Board following consent from the Italian Ministry of Health in accordance with the Italian law (D.lgs. 26/2014), following European Union policies (European and Economic Council Directive 86/609, OJL 358, December 12, 1987).

### **2.11. NS1-based Enzyme-Linked Immunosorbent Assay (NS1-based ELISA)**

Nunc Maxi Sorp Immuno-Plates (Thermo Fisher-Nunc, Roskilde, Denmark) were coated with 5 µg/ml of purified rNS1 antigens in 50 mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer pH 9.6 and incubated overnight at 4°C. Plates were washed with Phosphate-Buffered Saline (PBS) buffer, and non-specific binding sites were blocked with 2% milk in PBS for 45 min at room temperature (RT). After washing, 100 µl of 1:100 sera dilutions (or serial dilutions when it is indicated) of sera from immunized mice or 1:20 dilutions of sera from infected humans were added to each well.

For IgM detection, sera samples were incubated for 1 hour at 37°C, while for IgG detection, sera samples were incubated for 1 hour at RT. 100 µl/well of HRP-linked goat antibodies anti-mouse IgM/IgG or anti-human IgM/IgG were used (Sigma, 1:5000). In all cases, secondary antibodies were incubated for 1 hour at RT. After each antibody incubation, wells were washed three times with PBS 0.1% tween 20 (PBST). Signal was developed by adding 70 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma). The reaction was stopped by adding 30 µl of 2N H<sub>2</sub>SO<sub>4</sub> to each well. The optical density (OD) was measured at 450 nm (OD<sub>450</sub>) with an ELISA EnVision 2104 Multilabel Plate Reader (Perkin Elmer). The P/N ratio for both IgM/IgG NS1-based ELISA was obtained by dividing the OD<sub>450</sub> of the test specimen divided by the mean OD<sub>450</sub> of 16 negative control specimens. Experimental data by NS1-based ELISA presented in this thesis includes the average of two biological replicates.

### **2.12. Statistical analysis**

All statistical analyses and graphs were performed and generated using GraphPad Prism software. One-way ANOVA was used for analyses when needed (P values measured with one-way ANOVA, ns.  $p > 0.05$ . \*.  $p \leq 0.05$ . \*\*.  $p \leq 0.01$ . \*\*\*.  $p \leq 0.001$ ). The comparative receiver operating characteristic (ROC) curve analysis was used to calculate the optimal cut-off values of the P/N ratio (OD<sub>450</sub> of test specimen divided by the mean OD<sub>450</sub> of negative control specimens) for IgM/IgG detection by NS1-based ELISA.

The calculation of the cut-off value of the P/N ratios was done individually for each virus using sera samples from immunized mice or human sera. As an example, I show the calculation of the cut-off value for the detection of IgG antibodies from sera of mice immunized with a V5 construct of TBEV NS1 (Table 2.1).

		<b>Lowest P/N:</b>		<b>0.94</b>	
		<b>Highest P/N:</b>		<b>30.98</b>	
Bin range	IgG		Sensitivity	Specificity	False Positive
	T -	T +	P (T+   D+)	P (T-   D-)	P (T+   D-)
0.9	4	16	1	0	1
1	2	16	1	0.5	0.5
1.1	0	16	1	1	0
2	0	16	1	1	0
3	0	16	1	1	0
10	0	16	1	1	0
15	0	16	1	1	0
20	0	15	0.9375	1	0
25	0	11	0.6875	1	0
27	0	10	0.625	1	0
29	0	5	0.3125	1	0
31	0	0	0	1	0
Total:	4	16			

**Table 2.1: Calculation of the cut-off value of the P/N ratios for IgG detection from mice immunized with TBEV.**

I designed a bin range (0.9 to 31), including the lowest (0.94) and highest (30.98) P/N ratio values of four pre-immune sera samples (true negative, T-) and 16 sera from immunized mice (true positive, T+). Then, I analyzed the number of true negative or true positive samples that were positive from each group according to each value from the bin range (ei., if the cut-off is 0.9, all the true negative and true positive samples are classified as positive, 100% sensitivity and 0% specificity, respectively). Using these values, I calculated sensitivity (the probability (P) to detect true positive samples (T+) from individuals containing the disease (D+),  $P(T+ | D+)$ ); and the false-positive samples (the probability to detect true negative (T-) samples from individuals without the disease (D-),  $P(T+ | D-)$ ) of each specific value of the bin range. Having these values, I choose a value from the bin range (cut-off value of the P/N ratio) that gave the highest (100%, 1) sensitivity and specificity. In this case, the cut-off value for the detection of IgG antibodies from mice immunized with TBEV NS1 was 3, 100% sensitivity, and 100% specificity.



Reactivity	Species	Subtype	Source	Comments
Poly-Histidine-HRP	Mouse	Monoclonal	Sigma-Aldrich	1:2000 WB
V5	Mouse	Monoclonal	Provided by Dr. Oscar Burrone	1:10000 WB
human IgG ( $\gamma$ -chain specific)- HRP	Goat	Polyclonal	Sigma-Aldrich	1:5000 ELISA
Anti-Human IgM ( $\mu$ -chain specific)-HRP	Goat	Polyclonal	Sigma-Aldrich	1:5000 ELISA
Anti-Mouse IgG (whole molecule)- HRP	Goat	Polyclonal	Sigma-Aldrich	1:5000 ELISA
Anti-Mouse IgM ( $\mu$ -chain specific)-HRP	Goat	Polyclonal	Sigma-Aldrich	1:5000 ELISA

**Table 2.2. Antibodies used in this study.**

## RESULTS

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### 3. RESULTS

As a disclaimer, the reader is informed that some of the results included in this thesis were submitted for publication in *PLOS Neglected Tropical Diseases*. I am preparing an additional manuscript to submit for publication.

- Mora-Cárdenas E., Aloise C., Faoro V., Gašper N., Korva M., Caracciolo I., D'Agaro P., Avšič-Županc T., and Marcello A. (2019). Comparative specificity and sensitivity of NS1-based serological assays for the detection of flavivirus immune response. (Manuscript submitted for publication).
- #Caracciolo I., #Mora Cárdenas E., Aloise C., Carletti T., Segat L., Sole B., Chiarvesio A, TotisV., Avšič-Županc T., Mastrangelo E., Manfroni G., D'Agaro P., and Marcello A. (2019). Comprehensive response to usutu virus following first isolation in blood donors in the Friuli Venezia Giulia Region of Italy: development of recombinant NS1-based serology and sensitivity to antiviral drugs. (Manuscript submitted for publication).
- #Bamidele S., #Mora-Cárdenas E., Carletti T., Baba M., and Marcello A. Prevalence and locally undetected acute infections of flaviviruses in North-Eastern Nigeria. (Manuscript in preparation).

# Joint first authors, equal contribution, and alphabetic order.

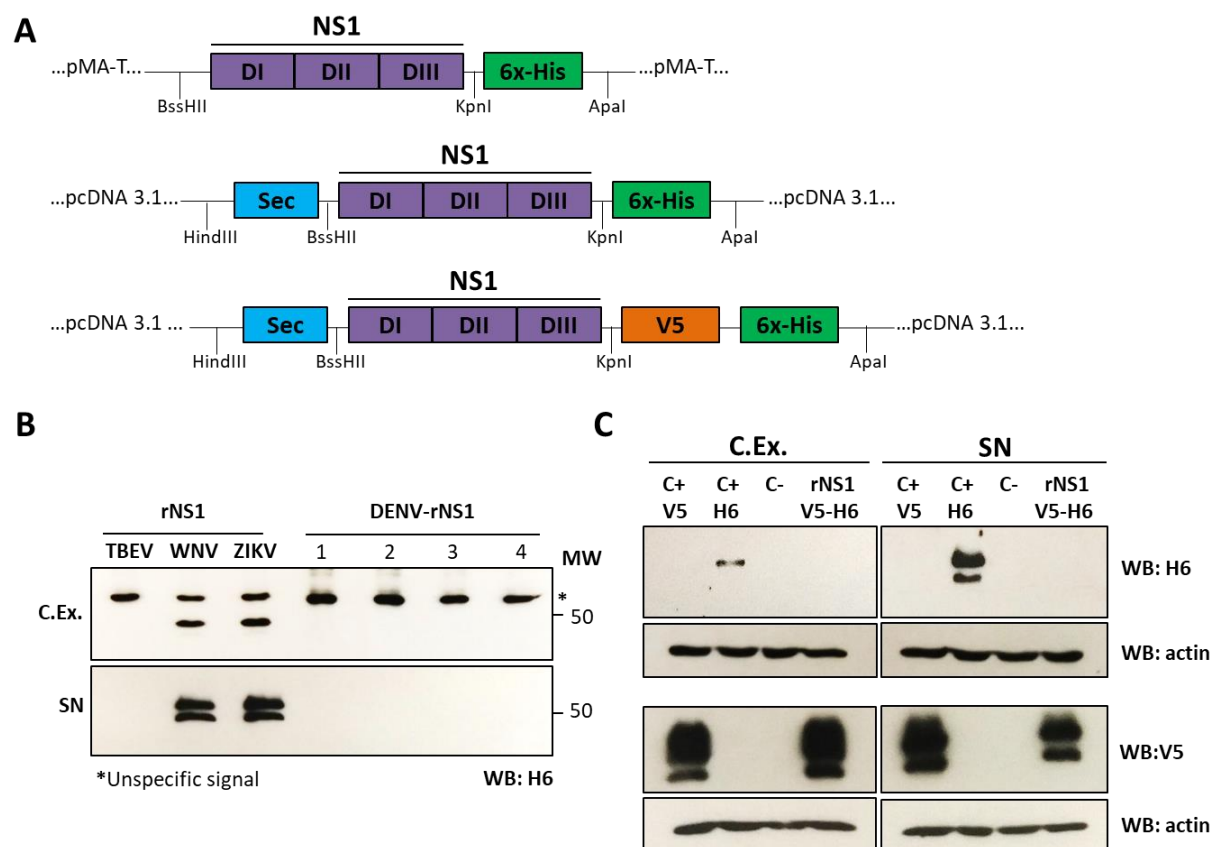
#### 3.1. Production of flavivirus Non-Structural Protein 1 (NS1)

The NS1 is a conserved non-structural protein among the flaviviruses. It is composed of a 352-amino-acids polypeptide constituting three different domains (I, II, and III) and plays several roles depending on its cellular location. NS1 is secreted from infected cells and represents one of the main targets for the immune response. With the aim to produce and purify fully antigenic, native recombinant NS1 (rNS1) of representative flaviviruses, I optimized the conditions to clone, express, and purify rNS1 proteins from the culture supernatant of transfected mammalian cells.

##### 3.1.1. Cloning and expression of His-tagged rNS1 protein of TBEV, WNV, ZIKV, and DENV1-4

Full-length wild type NS1 nucleotide sequences of TBEV, WNV, ZIKV, and DENV1-4 reference strains were optimized for the expression codons in *Mus musculus* and synthesized commercially. Only for WNV, two-point mutations were inserted (RQ10NK), which have been shown to increase the secretion of WNV-NS1 (Youn et al. 2010). Codon-optimized sequences

for mammalian expression of NS1 were obtained as synthetic genes in pMA-T vectors (Figure 3.1A, upper panel). The NS1 synthetic genes were cloned into the pcDNA3.1 expression vector and fused to a genomic fragment of a mouse heavy-chain immunoglobulin secretion signal (Sec) and, to a 6x-His-tag at the N-terminus and C-terminus, respectively (Figure 3.1A, middle panel). The Sec peptide signal was included to ensure the proper translocation of NS1 to the endoplasmic reticulum upon translation, while the 6x-His-tag was included for protein detection and purification.



**Figure 3.1. Cloning and expression of flavivirus Non-Structural Protein 1 (NS1).** **A)** Schematic representation of synthetic NS1 gene in the pMA-T vector (upper panel), sub-cloned into the pcDNA3.1 expression vector fused to an immunoglobulin leader sequence (Sec) at the N-terminus and the polyhistidine (6x-His) tag and/or the V5 tag at the C-terminus (middle and lower panel). **B)** Western Blot (WB) of cellular extracts (C.Ex.) and supernatants (SN) from HEK-293T cells transfected with the pcDNA3.1-Sec-NS1-6x-His constructs of the indicated viruses. TBEV, tick-borne encephalitis virus; WNV, west nile virus; ZIKV, zika virus, and DENV 1-4 corresponds to all dengue virus serotypes. The detection of unspecific bands are indicated with an asterisk **C)** WB of C.Ex. and SN from HEK-293T cells transfected with the pcDNA3.1-Sec-TBEVNS1-V5-6x-His construct. Blots show the analysis with an antibody against histidine tag (H6, upper panel) or V5 tag (lower panel). As a positive control (C+) cells were transfected with a pcDNA3.1-sec-WNVNS1-6x-His or pcDNA3.1-sec-WNVNS1-V5 construct, and mock-transfected cells were used as a negative control (C-). Each experiment was repeated at least 3 times with a single representative result shown.

The expression and secretion profiles of recombinant NS1 antigens were tested by western blot (WB) of cellular extracts and culture supernatants using a monoclonal antibody against histidine tag. WB of cellular extracts confirmed the expression only for WNV and ZIKV (Figure 3.1B, upper panel). Some unspecific bands around 55KDa were detected in all the samples. The comparable secretory phenotype was observed only for NS1 of WNV and ZIKV, while NS1 protein of TBEV and all four DENV serotypes were not detected in the supernatants (Figure 3.1B, lower panel).

In order to understand why the NS1 protein of TBEV and DENV1-4 were not detected in cell extract and supernatant, several experiments were performed using WNV-NS1, and TBEV-NS1 His-tagged constructs as controls (data not shown):

- A. Western blot using a specific antibody against TBEV-NS1 protein.
- B. Protein expression in HEK-293T cells by lowering culture temperature (28°C).
- C. Protein expression in different cell lines, including Vero and CHO cells (28°C and 37°C) and different transfection protocols (lipofectamine and polyethylenimine).
- D. Cloning of the NS1 gene with a different leader peptide (Bap31) to improve protein secretion.
- E. Cloning of the NS1 gene with a TEV cleavage site or Flag tag upstream the histidine tag. I did it in order to improve the exposure of the histidine tag. I thought the NS1 protein was not detected because the histidine tag was somehow hidden.
- F. I also checked the presence of the NS1 proteins by western blot loading the culture supernatant of transfected HEK-293T previously concentrated using 10K amicon ultra centrifugal filters.

In the first five cases (A-E), I did not get positive results with the TBEV-NS1 construct. However, when the culture supernatant from transfected cells was concentrated ten times, TBEV-NS1 protein was detected using an antibody against the histidine tag. These results could indicate a low expression of rNS1 of TBEV and DENV1-4.

In order to determine the level of expression and secretion of rNS1 proteins using two different antibodies, a set of oligos containing a V5 tag upstream, the histidine tag was designed and cloned into the pcDNA3.1 vector containing TBEV-NS1 gene to generate a V5-6x-His-tagged construct (Figure 3.1A, lower panel). HEK-293T cells were transfected with either a positive control (WNV-NS1 construct) containing V5 or histidine tag and the new construct of TBEV-

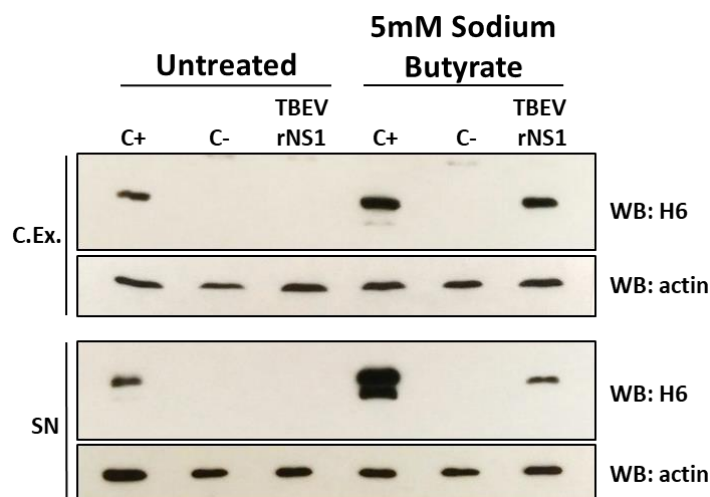
NS1 containing both tags. When the expression and secretion were evaluated by western blot against histidine tag, only the positive control was detected in both cellular extract and supernatant (Figure 3.1C, upper blot). Surprisingly, when the expression and secretion profiles of the same samples were analyzed using an antibody against the V5 tag, a meaningful signal in the positive control and NS1 of TBEV was obtained in both cellular extract and supernatant (Figure 3.1C, lower blot).

These results suggested that the expression levels of NS1 of TBEV were lower compared to WNV. The differences in the detection could be explained by the sensitivity of the two antibodies. The antibody against histidine tag is an anti-polyhistidine HRP-conjugated commercial antibody derived from the HIS-1 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from a mouse immunized with a polyhistidine-tagged fusion protein. On the other hand, the antibody against the V5 tag is a recombinant antibody fused to the human IgG Fc region produced and concentrated from culture supernatant of transfected HEK-293T mammalian cells.

### **3.1.2. Sodium butyrate enhances expression of flavivirus rNS1 protein**

Expression of recombinant NS1 proteins in mammalian cells is essential to preserve the glycosylation status and native conformation. However, the production of recombinant proteins requires the optimization of transient transfection protocols to have high yields. Previous studies have demonstrated that expression media can be supplemented with sodium butyrate and valproic acid to enhance protein expression in mammalian cells. These molecules block histone deacetylation leading to an increase in mRNA transcription levels. (Backliwal et al. 2008; Fan et al. 2005; Jeon and Lee, 2007; Wulhfard 2009).

To determine whether deacetylase inhibitors could increase the expression of rNS1, HEK-293T cells were transfected with TBEV-NS1 or WNV-NS1 (positive control) His-tagged constructs and treated with 5mM sodium butyrate 16 hours post-transfection. The expression and secretion of rNS1 were analyzed by western blot 24 hours after treatment. As is shown in Figure 3.2, the expression and secretion of both proteins were enhanced by sodium butyrate treatment. Similar results were obtained when the treatment was applied to cells transfected with all DENV-NS1 serotypes (data not shown). For this reason, all the transfections performed to purify rNS1 proteins included the treatment with 5mM of sodium butyrate.



**Figure 3.2. Sodium butyrate enhances the expression of rNS1 protein.** Western blot (WB) analysis of HEK-293T cells treated with 5 mM of sodium butyrate. The expression and secretion of rNS1 proteins were analyzed in cellular extract (C.Ex.) and supernatant (SN) using an antibody against histidine tag (H6). As a positive control (C+), cells were transfected with a pcDNA3.1-sec-WNVNS1-6x-His construct, and mock-transfected cells were used as a negative control (C-). The experiment was repeated at least 3 times with a single representative result shown.

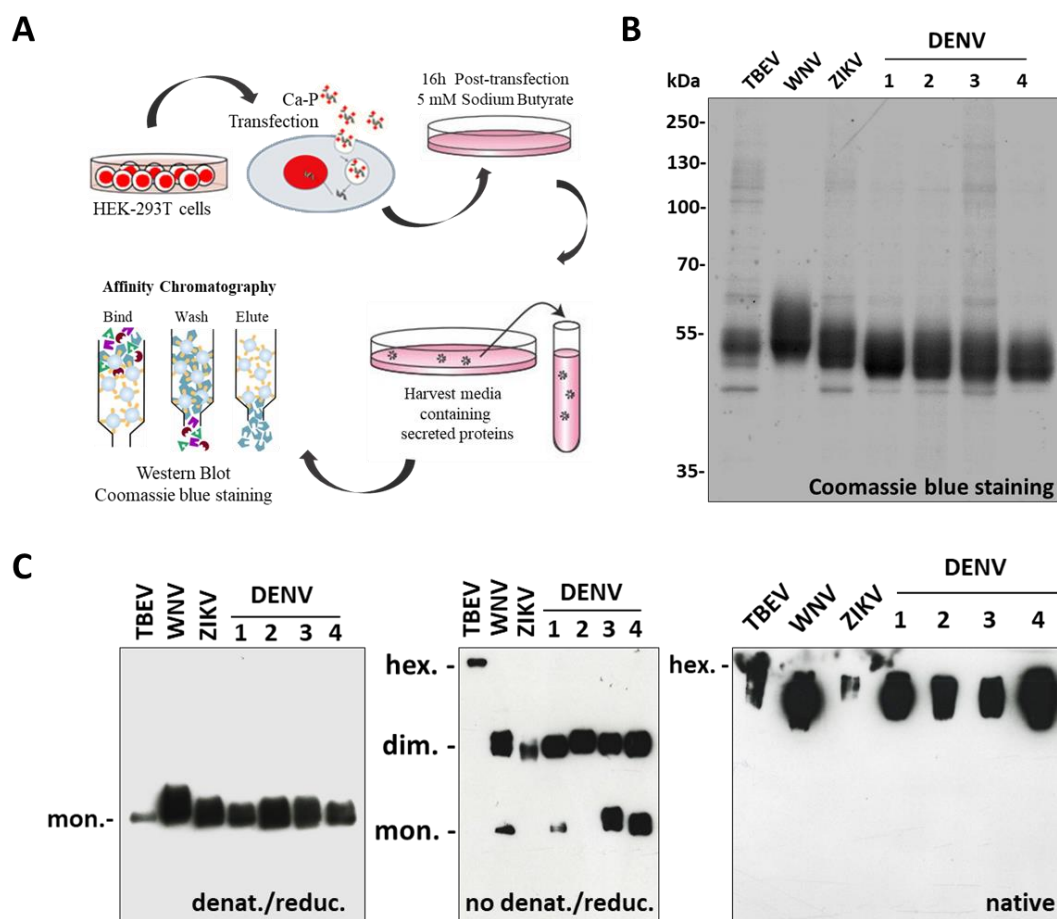
### 3.1.3. Purification of rNS1 protein of TBEV, WNV, ZIKV, and DENV1-4

Calcium phosphate transfection of the NS1 full-length gene of TBEV, WNV, ZIKV, and DENV 1-4 was optimized to produce and purify rNS1 proteins in large-scale (Figure 3.3A). Briefly,  $5.0 \times 10^6$  HEK-293T cells/150mm plate were cultured with DMEM media supplemented with 10% fetal bovine serum (FBS). The transfected cells were washed twice with PBS 16h following transfection and then incubated 24h at 37°C in DMEM supplemented with 5 mM of sodium butyrate and without FBS. Purification of rNS1 proteins present in the supernatant was carried out via nickel-affinity chromatography. Secreted rNS1 proteins of TBEV, WNV, ZIKV, and DENV 1-4 were successfully purified and analyzed by western blot and coomassie blue staining. In each purification I got around 700µg of NS1 proteins from the supernatant of 20 plates. Some antigens such as NS1 derived from ZIKV were purified in larger amounts, while NS1 derived from DENV3 was more difficult to purify.

### 3.1.4. Purity and oligomeric status of purified rNS1 proteins

Densitometric analysis of coomassie-stained polyacrylamide gel showed between 87 – 92% purity for each purified protein (Figure 3.3B). The oligomeric status of the purified proteins was analyzed by western blot (WB) using an antibody against the 6x-His-tag. WB of denatured purified rNS1 proteins diluted in reducing Laemmli sample buffer (LB) showed a single band for

TBEV, meanwhile, for ZIKV and DENV1-4, a thicker band (overlapping of 2 or 3 bands depending on the glycosylation status of NS1) was observed. In all cases, each band corresponds to the 50-55 kDa monomeric form of NS1 (Figure 3.3C, left panel). When the WB was performed in non-denaturing/non-reducing conditions, mainly dimeric forms of NS1 protein were observed. Interestingly, TBEV-NS1 protein was uniquely stable as hexamers (Figure 3.3C, middle panel). When the purified rNS1 proteins were analyzed under native conditions, all of them were composed of high molecular weight oligomers (Figure 3.3C, right panel).

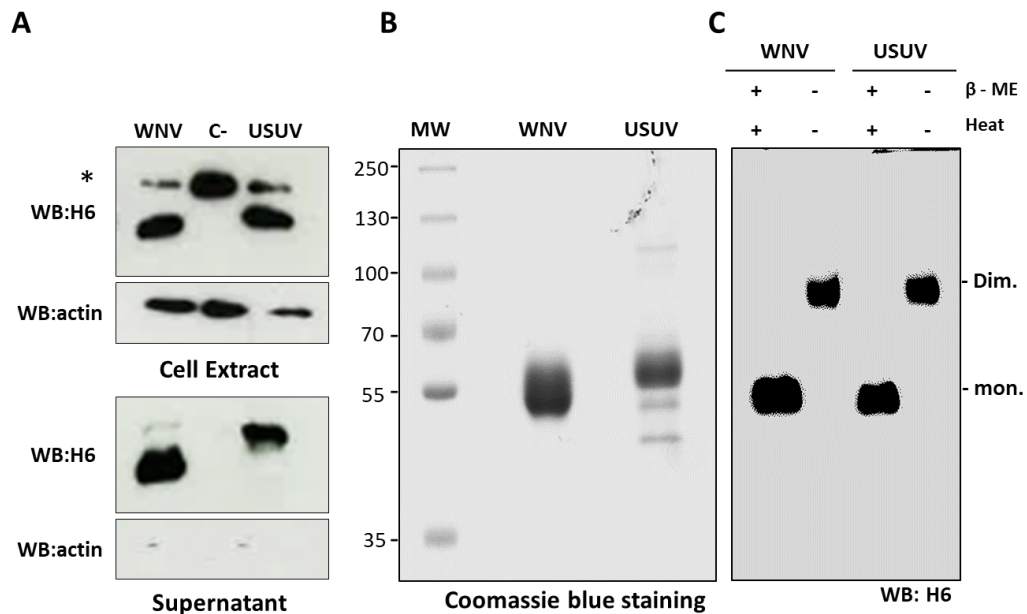


**Figure 3.3. Purification and western blot analysis of recombinant flavivirus Non-Structural Protein 1 (sNS1).** **A)** Schematic representation of all the steps followed for the expression of 6x-His-tagged constructs in transfected HEK-293T cells and purification of rNS1 proteins by affinity chromatography. **B)** coomassie blue staining of purified proteins: 2  $\mu$ g of purified rNS1 protein was loaded in each lane. TBEV, tick-borne encephalitis virus; WNV, west Nile virus; ZIKV, zika virus, and DENV 1-4 corresponds to all DENV serotypes. **C)** Western blot (WB) analysis of purified rNS1. Left panel, purified rNS1 proteins diluted in reducing Laemmli sample buffer (LB) and denatured by boiling using an antibody against the histidine tag (WB: H6). Middle panel, WB of rNS1 performed in non-denaturing/non-reducing conditions (without heating the proteins and LB without 2- $\beta$ ME). Right panel, WB of purified rNS1 proteins analyzed by WB under native conditions (without heating the proteins; without 2- $\beta$ ME and SDS). rNS1 monomers (mon), dimers (dim), and hexamers (hex) are indicated. Each experiment was repeated at least 3 times with a single representative result shown.



### 3.1.5. Cloning, expression, and purification of USUV-rNS1 protein

As I mentioned in the introduction, usutu virus (USUV) is a re-emerging virus with increasing spatial distribution in Europe (Cheng et al. 2018). Diagnosis is mainly made by molecular assays because of a relevant limitation of available serological tests. So far, there is only one commercial test based on the detection of IgG antibodies against envelope protein (Euroimmun, Germany). Therefore, I applied the same strategy mentioned above to clone, express, and purify rNS1 of USUV from transfected HEK-293T cells.



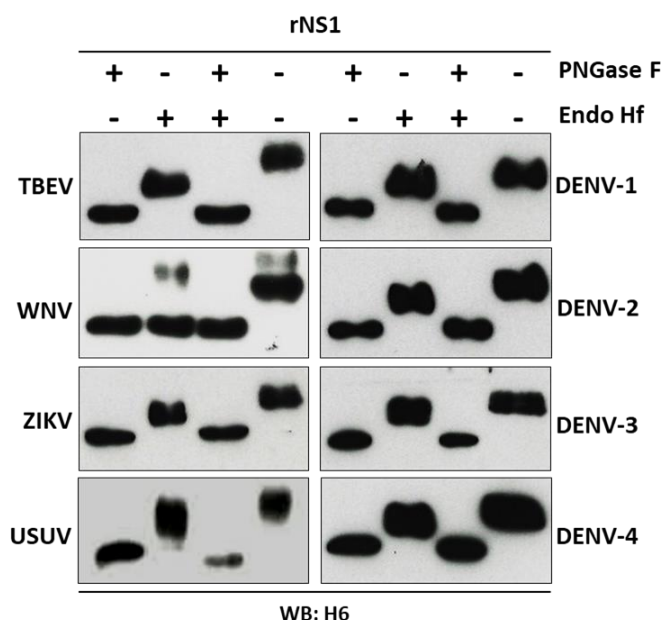
**Figure 3.4. Expression and purification of rNS1 of usutu Virus (USUV).** **A)** Western Blot (WB) analysis of cellular extracts and culture supernatant from HEK-293T cells transfected with the pcDNA3.1-Sec-USUVNS1-6x-His construct. Unspecific bands are indicated with an asterisk **B)** coomassie blue staining of purified USUV/WNV-rNS1: 2  $\mu$ g of purified protein was loaded. **C)** WB analysis of purified rNS1 of USUV in non-denaturing/non-reducing conditions (without heating the proteins and Loading Buffer without 2- $\beta$ ME). rNS1 monomers (mon) and dimers (dim) are indicated. As a positive control, cells were transfected with a pcDNA3.1-sec-WNVNS1-6x-His construct, and mock-transfected cells were used as a negative control (C-). Each experiment was repeated at least 3 times with a single representative result shown.

As shown in the scheme in figure 3.1A, middle panel, I cloned the full-length sequence of the USUV-NS1 gene in a pcDNA3.1 vector containing a Sec leader peptide at the N-terminus and 6x-His-tag at the C-terminus. The expression and secretion of rNS1 were analyzed by WB in both, cell extract and culture supernatant using an antibody against histidine tag (Figure 3.4A). I also performed a densitometric analysis of a coomassie stained gel to check the purity of the purified protein (Figure 3.4B). It ranged between 87 to 90%, similar purity to commercially

available flavivirus NS1 proteins (the native antigen company, UK). Additionally, I checked the oligomeric status of purified USUV-NS1 by WB. A thick band corresponding to the 50-55 kDa monomeric form of NS1 was observed when the sample was boiled and prepared in reducing Laemmli sample buffer, while only dimeric forms of NS1 were observed when the WB was performed in non-denaturing/non-reducing conditions (Figure 3.4C). Comparable results were obtained for WNV NS1 used as a control.

### 3.1.6. Purified flavivirus rNS1 proteins preserve their glycosylation status

NS1 gene of all flaviviruses encodes a 352-amino-acid polypeptide with two or three conserved N-glycosylation sites resulting in a monomer with a variable molecular weight of 46 – 55 kDa (Muller and Young 2013). Three N-glycosylation sites (Asn 130, 207 and 175) have been widely described for NS1 protein in all members of the Japanese encephalitis subgroup (including WNV), while only the first 2 glycosylation sites are perfectly conserved among all mosquito-borne flaviviruses, including USUV, ZIKV and all DENV serotypes (Bakonyi et al. 2004; Pryor and Wright 1994). In the case of TBEV, only the potential N-glycosylation site at amino acid 207 has been well documented with other two potential sites at positions 85 and 223, which are not related to those of mosquito-borne flaviviruses (Mandl, Heinz, and Kunz 2004).



**Figure 3.5. Endoglycosidase analysis of purified rNS1 proteins.** All recombinant NS1 (rNS1) proteins of TBEV, WNV, ZIKV, USUV, and all four DENV serotypes (DENV1-4) were treated (+)/or not (-) with PNGase F and/or Endo -Hf enzymes for 1.5 h at 37 °C. Western blot (WB) analysis was assessed by 10% SDS-PAGE under standard conditions using an anti-6xHis-tag monoclonal antibody (WB: H6). Each experiment was repeated at least 3 times with a single representative result shown.

In order to assess the glycosylation status of the purified proteins, I performed an enzymatic deglycosylation assay with peptide-N-glycosidase F (PNGase F, which cleaves all sugar moieties from high mannose, hybrid, and complex oligosaccharides) and/or endoglycosidase Hf (Endo Hf, which removes high-mannose-content sugars). The presence of N-glycans was analyzed by a shift in band migration compared to undigested proteins (Figure 3.5). The pattern of deglycosylated proteins with PNGase F was comparable for all rNS1 proteins. Digested proteins were observed as lower band compared to undigested proteins used as a control.

In particular, all antigens, except for WNV rNS1, show a migration profile in the Endo H treated samples that is compatible with the pattern described for DENV NS1, with an Endo H resistant complex type glycan at N130 and an Endo H sensitive high mannose simple glycan at N207 (Pryor and Wright 1994). The only variation to the theme is for WNV NS1 that shows complete digestion with Endo H (Figure 3.5). Together, these results confirmed the presence of N-linked glycans in all rNS1 proteins purified from the supernatant of transiently transfected HEK-293T cells.

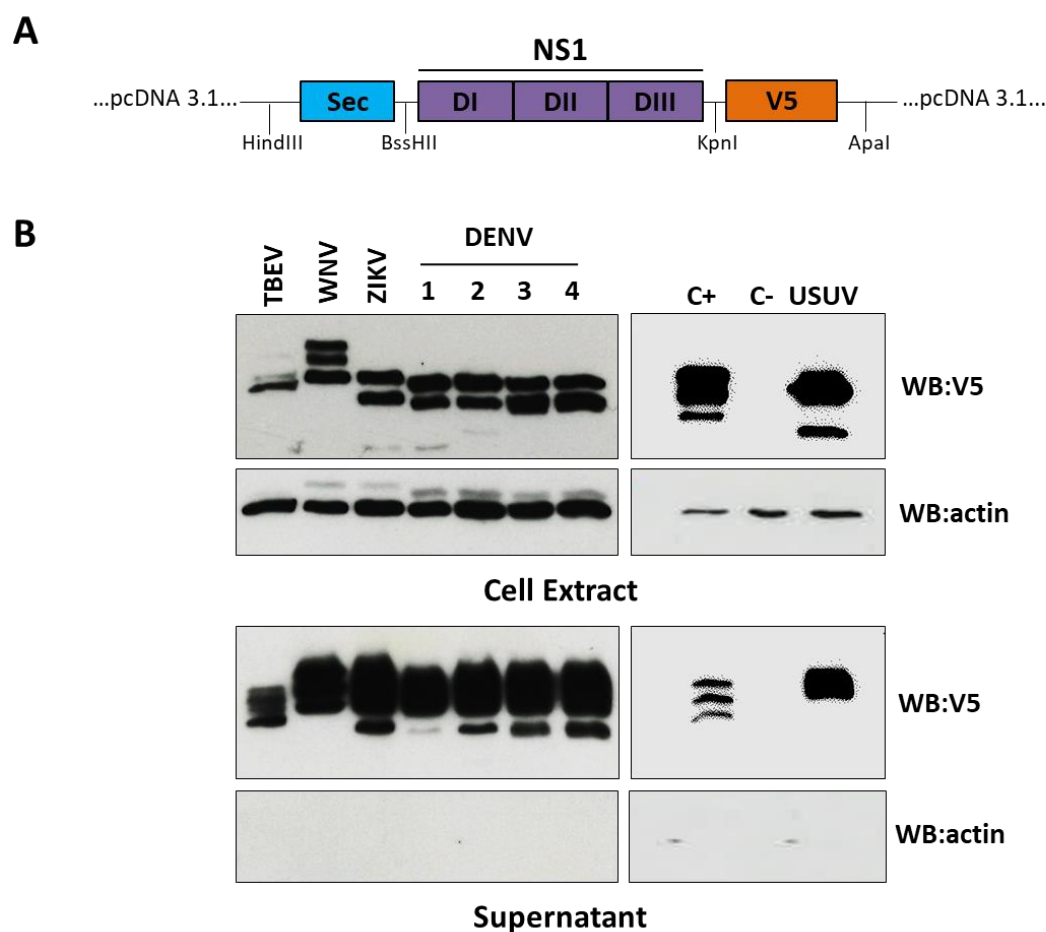
### **3.2. Validation of the immunological properties of purified rNS1 antigens by NS1-based ELISA**

High level of cross-reactivity among structural virion proteins that are generally used as antigens for antibody detection is one of the main problems in the diagnosis of flavivirus infections (Koraka et al. 2002; Sa-Ngasand et al. 2006; Singh et al. 2018). It has been very well reported, especially in endemic areas where the population contains flavivirus-reactive antibodies due to natural infections or vaccination (Priyamvada et al. 2016; Rabe et al. 2016). Therefore, to validate the immunological properties of purified rNS1 proteins using defined positive sera samples for a single flavivirus, and to optimize an ELISA protocol for the detection of IgM/IgG antibodies against His-tagged rNS1 proteins (NS1-based ELISA), I used sera samples from immunized mice with plasmids encoding V5-tagged NS1 gene.

#### **3.2.1. Cloning and expression of V5-tagged flavivirus NS1 proteins**

I cloned the full-length NS1 nucleotide sequence of TBEV, WNV, ZIKV, USUV, and all DENV serotypes (1- 4) in a pcDNA3.1 vector containing a Sec leader peptide at the N-terminus and a V5 tag downstream the NS1 gene (Figure 3.6A). Concerning previous His-tagged constructs used for protein purification, the only difference is the V5 tag. I changed the tag in order to

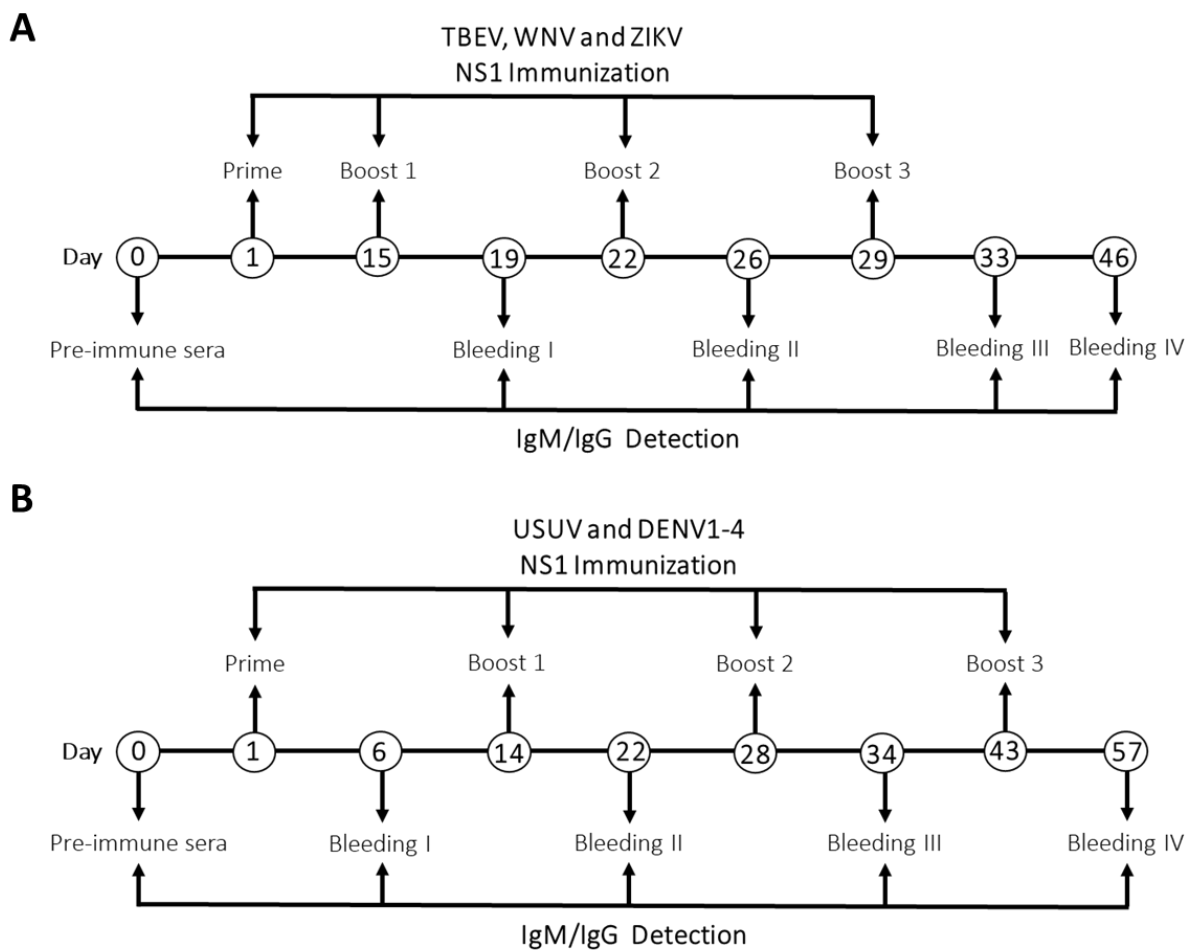
avoid the development of antibodies against the histidine tag that could recognize purified His-tagged recombinant proteins. I checked the expression and secretion of V5-tagged rNS1 proteins in cell extract and culture supernatant of transiently transfected HEK-293T cells by western blot using an antibody against the V5 tag (Figure 3.6B). According to the glycosylation status, one, two, or three different bands were detected in cell extracts for TBEV, ZIKV/USUV/DENV1-4, and WNV, respectively. Thicker bands due to the overlapping of more than one band and strong signal were detected in the supernatants.



**Figure 3.6. Cloning and expression of V5-tagged constructs for mice immunization. A)** Schematic representation of pcDNA3.1-sec-NS1-V5 constructs used for mice immunization. Sec corresponds to an immunoglobulin leader sequence at the N-term; V5 is a tag cloned at the C-term. **B)** Western blot (WB) analysis of V5-tagged recombinant NS1 proteins of TBEV, WNV, ZIKV, USUV, and DENV1-4 in cell extract and culture supernatant of transiently transfected HEK-293T cells. As a positive control, cells were transfected with a pcDNA3.1-sec-WNVNS1-V5 construct, and mock-transfected cells were used as a negative control (C-). Actin blots were included as a loading control for cell extracts and of clean supernatants. Each experiment was repeated at least 3 times with a single representative result shown.

### 3.2.2. Gene gun-mediated intradermal DNA immunization

After I confirmed the expression and secretion of V5-tagged rNS1 proteins, different groups of BALB/c mice (4 mice each (Rx, Lx, RxLx, and unmarked ear) for TBEV, WNV, ZIKV, USUV, and DENV1-4) were intradermally immunized following two different schemes based on prime-boost immunization. The first group of mice was immunized with NS1 V5-tagged construct of TBEV, WNV or ZIKV at days 1, 15, 22 and 29, and sera samples were collected at days 0 (pre-immune sera), 19 (bleeding I), 26 (bleeding II), 33 (bleeding III) and 46 (bleeding IV) (Figure 3.7A).



**Figure 3.7. Schemes of mice immunization with V5-tagged NS1 constructs.** 4 different BALB/c mice for each virus were immunized by gene gun technology. **A)** scheme for mice immunization with V5-tagged constructs encoding for NS1 protein of TBEV, WNV, and ZIKV. **B)** scheme for mice immunization with USUV and DENV1-4 V5-tagged constructs. Blood samples were collected by sub-mandibular puncture before immunization (pre-immune sera) and 5-7 days after each boost (bleeding I, II, III, and IV). Sera samples were collected and stored at -20°C until use.

Since there is an increase in the number of reports showing that high levels of IgG antibodies can affect the detection of IgM antibodies (Wong et al. 2004), I followed a different scheme

for USUV and DENV1-4 NS1 immunization. The second group of mice was immunized at fourteen days intervals and, importantly, sera samples were collected earlier (compared to the first group) after the first immunization (days 0, 6, 22, 34, and 57) (Figure 3.7B).

### 3.2.3. Detection of IgM and IgG antibodies by NS1-based ELISA from sera of immunized mice

Sera samples from immunized mice with NS1 V5-tagged construct of TBEV, WNV, ZIKV, USUV, and DENV1-4 were used for the detection of IgM/IgG antibodies by NS1-based ELISA. Briefly, 96-well ELISA plates were coated with purified rNS1 antigens and incubated with 1:100 sera dilution for 1h at 37°C for the detection of IgM antibodies; while the detection of IgG antibodies was performed at room temperature (RT). I used a peroxidase-labeled goat anti-mouse secondary antibody and TMB (3,3',5,5'-tetramethylbenzidine) to develop the assay. The optical density was measured at 450 nm (OD<sub>450</sub>).

The results are presented as a positive to negative (P/N) ratio calculated by dividing the OD<sub>450</sub> of test specimen by the OD<sub>450</sub> of the negative control (mean of pre-immune sera). Cut-off values of P/N ratios were calculated based on the comparative receiver operating characteristic (ROC) curve analysis (detailed description in materials and methods). The optimal cutoff values for IgM and IgG detection from immunized mice fell at 1.2 and 3, respectively (Table 1). Each cut-off was selected based on the P/N ratio value, which gave 100% sensitivity and specificity.

	<b>rNS1 antigens</b>							
	<b>TBEV</b>	<b>WNV</b>	<b>ZIKV</b>	<b>USUV</b>	<b>DENV-1</b>	<b>DENV-2</b>	<b>DENV-3</b>	<b>DENV-4</b>
<b>IgM</b>	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
<b>IgG</b>	3	3	3	3	3	3	3	3

**Table 3.1. Optimal cut-off values of the P/N ratio for IgM and IgG antibody detection from the sera of immunized mice.** rNS1: recombinant non-structural protein 1, TBEV: tick-borne encephalitis virus, WNV: west Nile virus, ZIKV: zika virus, USUV: usutu virus, DENV1-4: dengue virus serotype 1, serotype 2, serotype 3 and serotype 4.

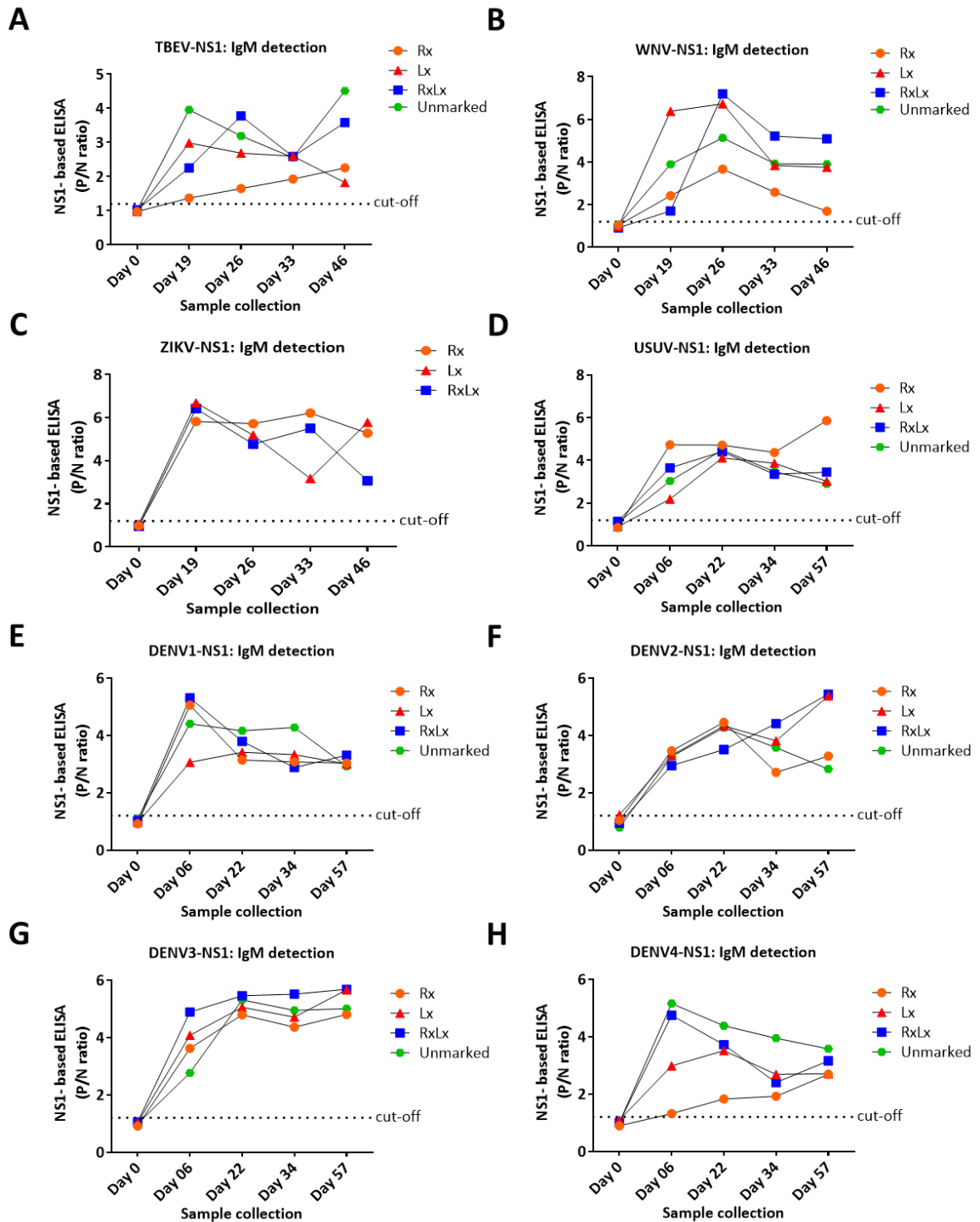
### **3.2.3.1. Detection of IgM antibodies**

I checked the presence of IgM antibodies in all 4 different immunized mice (Rx, Lx, RxLx, and unmarked ear) for each NS1 of TBEV, WNV, ZIKV, USUV and DENV1-4 (Figure 3.8 A to H). As expected, IgM P/N ratios below the cut-off were obtained in all pre-immune sera samples. Independently of the immunization scheme followed, I observed comparable IgM ratios above the cut-off in all the samples (bleeding I to IV) from immunized mice with TBEV, WNV, ZIKV, USUV, and DENV1-4. IgM P/N ratios were more consistent between mice immunized with ZIKV, USUV, or DENV1-3, while more variability between mice immunized with TBEV, WNV, or DENV4 was observed.

### **3.2.3.2. Serologic cross-reactivity of IgM antibodies to flavivirus rNS1 antigens**

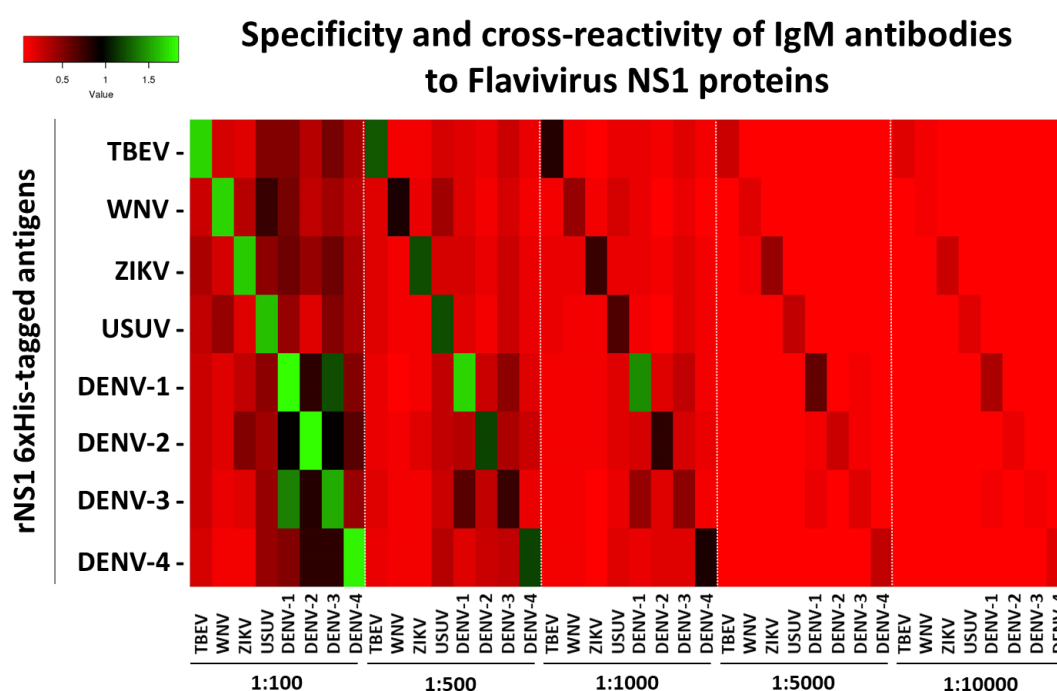
I checked the specificity of each purified rNS1 protein to detect IgM antibodies from sera samples of mice immunized with either TBEV, WNV, ZIKV, USUV, or DENV1-4. I selected the second bleeding (bleeding II) of each NS1-specific serum that gave the highest P/N ratio when they were tested to the homologous NS1 protein (i.e., I selected the bleeding II (day 26) of the unmarked mouse immunized with NS1 V5-tagged construct of TBEV). The ability of each purified rNS1 protein to detect specific IgM antibodies was tested using several dilutions (1:100 to 1:10:000) of each serum sample. The results are represented in a heatmap in Figure 3.9.

Specific IgM antibodies from the serum of each immunized mouse were detected up to a 1:1000 dilution, and for some of them, the signal was still detected up to 1:10000 dilution. rNS1 protein of TBEV, ZIKV, USUV, or DENV4 allows the specific detection of IgM antibodies from mice immunized with homologous NS1 protein without the detection of cross-reactive antibodies from mice immunized with heterologous NS1 proteins. rNS1 protein of WNV showed the detection of some cross-reactive antibodies from the serum of a mouse immunized with USUV up to 1:500 dilution. However, the detection of specific antibodies was observed when the serum was more diluted.



**Figure 3.8. Detection of IgM antibodies by NS1-based ELISA using sera samples from immunized mice. A-H)** Sera samples from 4 different immunized mice with NS1 V5-tagged construct of TBEV, WNV, ZIKV, USUV, and DENV1-4 were collected at different time points (indicated by day of sample collection). Each serum sample (1:100 dilution) was reacted to the homologous rNS1 antigen in ELISA plates coated with purified antigens (5 $\mu$ g/mL). The results are presented as positive/negative (P/N) ratio (OD<sub>450</sub> of test specimen divided by the mean OD<sub>450</sub> of negative control specimens). Optimal cut-off values of the P/N ratio were calculated based on the comparative receiver operating characteristic (ROC) curve analysis. Cut-off values for IgM fell at 1.2. Mice were differentiated with a mark in the right ear (Rx), left (Lx), both ears (RxLx), or without the mark (unmarked). Each ELISA result includes an average of two biological replicates.





**Figure 3.9. Specificity of purified rNS1 proteins to detect IgM antibodies from the sera of immunized mice.** Several serum dilutions (1:100 to 1:10000) from an immunized mouse with NS1 V5-tagged constructs of TBEV, WNV, ZIKV, USUV, or DENV1-4 were analyzed in ELISA plates coated with homologous and heterologous rNS1 6xHis-tagged antigens (5µg/mL). Determination of positive and negative samples was based on the cut-off values shown in table 3.1, and reactivity intensity is shown according to the colorized scale with green strongest, black in-between, and red weakest. Represented results include an average of two biological replicates.

As showed in figure 3.9 the rNS1 proteins of DENV1-3 serotypes allows the detection of specific IgM antibodies with very low detection of cross-reactive antibodies from sera of immunized mice with heterologous NS1 of TBEV, WNV, ZIKV, and USUV. Some cross-reactive IgM antibodies were detected as follows: rNS1 protein of DENV1 detected cross-reactive antibodies from mice immunized with NS1 of DENV2 or DENV3 up to 1:500 dilution. rNS1 protein of DENV2 strongly detected cross-reactive antibodies from mice immunized with NS1 of DENV1 or DENV3 and some cross-reactive antibodies from mice immunized with NS1 of DENV4. However, the detection of specific antibodies was evident, starting from 1:500 dilution. Finally, the rNS1 protein of DENV3 mainly detected cross-reactive antibodies from a mouse immunized with NS1 of DENV1 up to 1:1000 and low cross-reactive antibodies with NS1 of DENV2 in the first serum dilution.

### 3.2.3.3. Detection of IgG antibodies

I also checked the presence of IgG antibodies in all four different mice immunized (Rx, Lx, RxLx, and unmarked ear) with NS1 V5-tagged constructs of TBEV, WNV, ZIKV, USUV, and DENV1-4

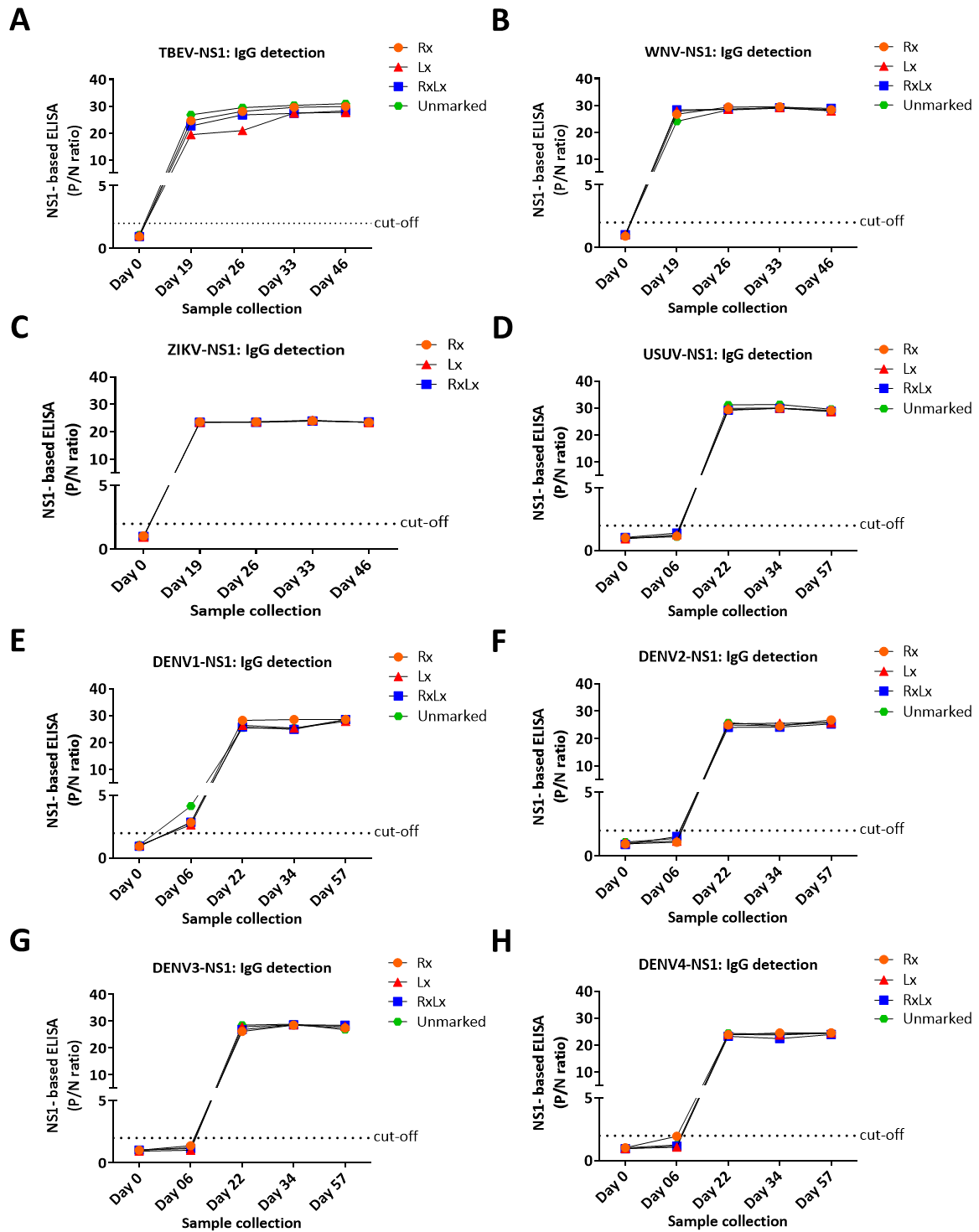
(Figure 3.10 A-H). The IgG P/N ratios were very consistent between mice immunized with the same NS1 V5-tagged construct and comparable between different constructs.

As expected, IgG P/N ratios below the cut-off were detected in all pre-immune sera samples (day 0). Based on the two different schemes followed for the mice immunization, some differences were observed in the follow-up samples. IgG P/N ratios above the cut-off were obtained from the bleeding I (day 19) in all sera samples from mice immunized with TBEV, WNV, or ZIKV. However, due to the short period between the immunization with USUV/DENV1-4 and the first sample collection (5 days), IgG P/N ratios above the cut-off were only obtained after the bleeding II (day 22).

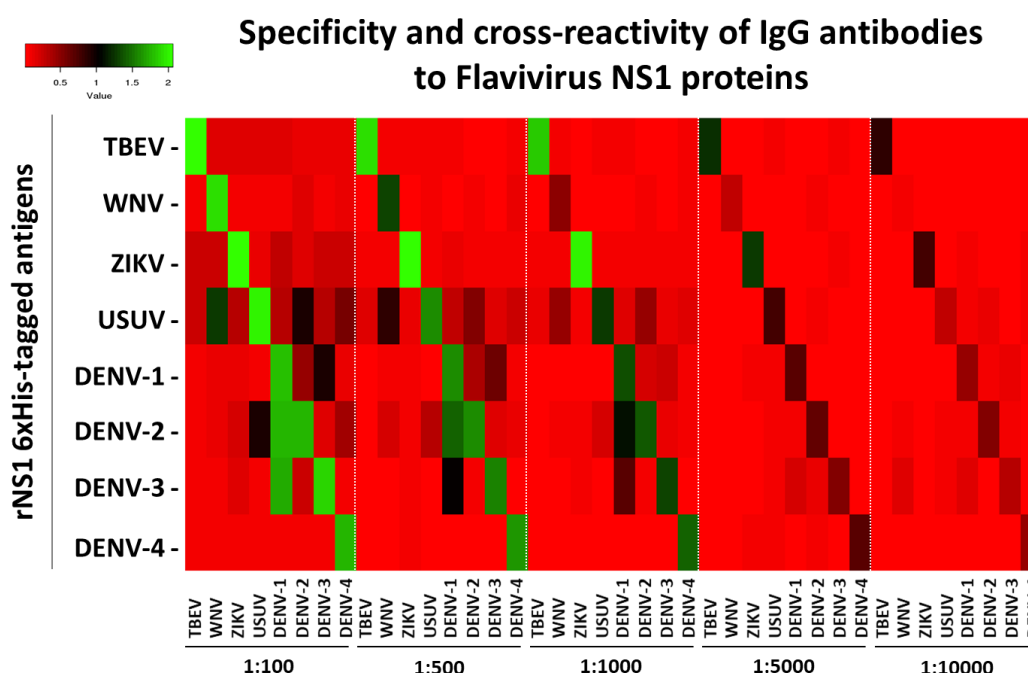
#### **3.2.3.4. Serologic cross-reactivity of IgG antibodies to flavivirus rNS1 antigens**

As described above for the detection of specific IgM antibodies, I checked the ability of each purified rNS1 protein to detect specific IgG antibodies from several sera dilutions (1:100 to 1:10:000) of mice immunized with homologous or heterologous NS1 protein of TBEV, WNV, ZIKV, USUV or DENV1-4. The results are represented in a heatmap in Figure 3.11. In almost all cases, rNS1 proteins allowed the specific detection of IgG antibodies up to 1:10000 serum dilution, except NS1-WNV up to 1:5000. I did not detect cross-reactive antibodies developed against heterologous NS1 antigens with rNS1 protein of TBEV, WNV, ZIKV, and DENV4. However, some cross-reactive antibodies were detected using rNS1 proteins of USUV and DENV1-3 as follow:

- rNS1 protein of USUV mainly detected cross-reactive IgG antibodies from sera of mice immunized with NS1 of WNV or DENV2, while some cross-reactive DENV4 IgG antibodies were only detected up to 1:500 dilution.
- rNS1 protein of DENV1 strongly detected cross-reactive IgG antibodies from the serum of a mouse immunized with NS1 of DENV2 and DENV3 up to 1:500 dilution.
- rNS1 protein of DENV2 detected highly cross-reactive IgG antibodies from the serum of a mouse immunized with NS1 of DENV1 up to 1:1000, and minor cross-reactive antibodies from a mouse immunized with NS1 of USUV up to 1:500 dilution.
- rNS1 protein of DENV3 detected cross-reactive IgG antibodies from the serum of a mouse immunized with NS1 of DENV1 up to 1:5000 dilution.



**Figure 3.10. Detection of IgG antibodies by NS1-based ELISA using sera samples from immunized mice. A-H)** Sera samples from 4 different immunized mice with NS1 V5-tagged constructs of TBEV, WNV, ZIKV, USUV, DENV1-4 were collected at different time points (indicated by day of sample collection). Each serum sample (1:100 dilution) was reacted to the homologous rNS1 antigen in ELISA plates coated with purified antigens (5 $\mu$ g/mL). The results are presented as positive/negative (P/N) ratio (OD<sub>450</sub> of test specimen divided by the mean OD<sub>450</sub> of negative control specimens). Optimal cut-off values of the P/N ratio were calculated based on the comparative receiver operating characteristic (ROC) curve analysis. Cut-off values for IgG fell at 3. Mice were differentiated with a mark in the right ear (Rx), left (Lx), both ears (RxLx), or without the mark (unmarked). Each ELISA result includes an average of two biological replicates.



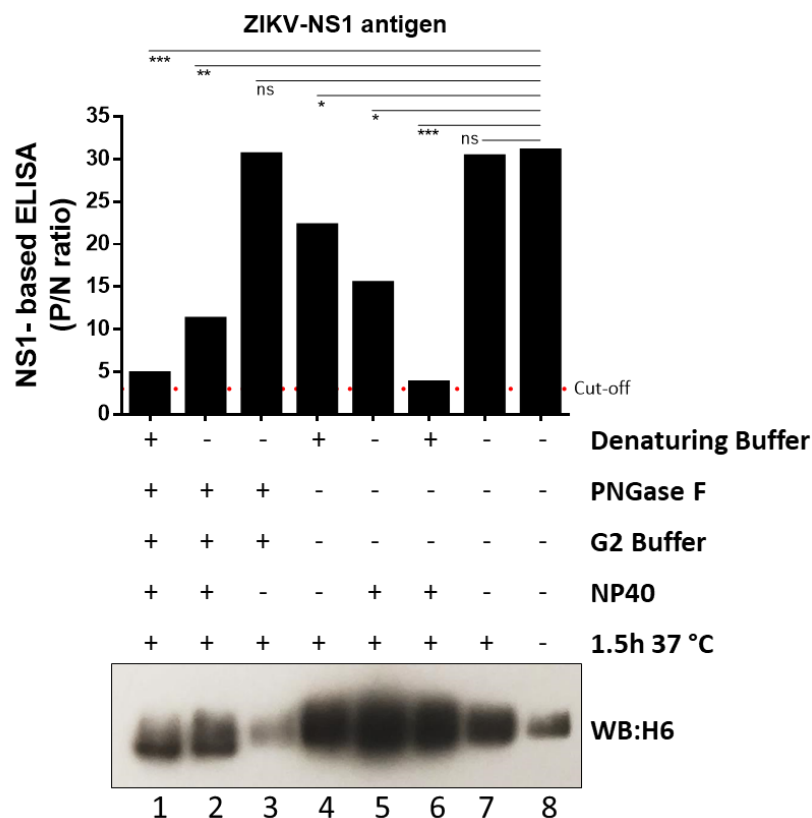
**Figure 3.11. Specificity of purified rNS1 proteins to detect IgG antibodies from the sera of immunized mice.** Several serum dilutions (1:100 to 1:10000) from an immunized mouse with NS1 V5-tagged construct of TBEV, WNV, ZIKV, USUV, or DENV1-4 were analyzed in ELISA plates coated with homologous and heterologous rNS1 6xHis-tagged antigens (5µg/mL). Determination of positive and negative samples was based on the cut-off values shown in table 3.1, and reactivity intensity is shown according to the colorized scale with green strongest, black in-between, and red weakest. Represented results include an average of two biological replicates.

### 3.3. The native conformation of NS1 antigens is required for antibody detection

In order to analyze the role of N-linked glycans present in flavivirus NS1 proteins (complex glycans at N-130, and high mannose glycans at N-207) for the detection of specific antibodies, I did an NS1-based ELISA using deglycosylated ZIKV NS1 with PNGase F (removes complex glycans and high mannose glycans) and/or Endo Hf (removes high mannose glycans). First, I performed an assay under denaturing conditions following the protocol according to the manufacturer's instructions (New England Biolabs). It includes a denaturing step at 95°C for 10 min (heated), followed by the digestion for 1.5 hours at 37°C. The digestion sensitivity was measured through observation of a shift in band migration compared to undigested proteins by western blot using an antibody directed to the histidine tag. ZIKV NS1 was sensitive to digestion by PNGase F and Endo Hf when the protein was treated with a single or both enzymes, indicating the removal of the N-glycans. A higher band corresponding to the



Under these conditions, the digestion with Endo Hf seems to be more efficient compared to PNGase F. However, in both cases can be noticed a band that shows different grades of efficiency compared to undigested proteins (Figure 3.12C). I used this partially digested ZIKV NS1 under native conditions for the detection of IgG antibodies from the same serum sample mentioned above (Figure 3.12D). When ZIKV NS1 was treated with Endo Hf, IgG antibodies were detected with comparable sensitivity than undigested proteins. However, digestion with PNGase F alone or in combination with Endo Hf strongly affected the detection of IgG antibodies.



**Figure 3.13. Native conformation of NS1 antigens is required for antibody detection.** Western blot analysis (lower panel) of digested ZIKV NS1 with PNGase F under native conditions with all the reagents or a combination of some of them (lane 1-8). The same treated proteins were used for the detection of IgG antibodies by NS1-based ELISA. -, untreated; +, treated. Each experiment was repeated at least 3 times with a single representative result shown. Statistics as already described in the legend of Fig. 3.12.

Taken together, these results could suggest that the removal of complex glycans and high mannose glycans with PNGase F probably affects some conformational epitopes required for IgG reactivity. However, since I observed in figure 3.12B that the native conformation of ZIKV NS1 is required for IgG detection, I checked carefully the effect of the reagents used in the deglycosylation assay by western blot and NS1-based ELISA assay (Figure 3.13). As shown

before, ZIKV NS1 was partially digested (lane 1 and 2) when it was done under native conditions and adding all the reagents (denaturing buffer (not in lane 2), PNGase F enzyme, G2 buffer, NP40, and digestion for 1.5h at 37°C). However, the protein was not digested when the enzyme was added only with the buffer (lane 3) or when the protein was treated with single reagents and without the enzyme (lane 4-8).

The same proteins analyzed by western blot were used for the detection of IgG antibodies by NS1-based ELISA (Figure 3.13, upper panel). Only when ZIKV NS1 was treated for 1.5h at 37°C with PNGase F + G2 buffer (lane 3), IgG antibodies were detected with the same sensitivity as proteins that were only incubated or not for 1.5h at 37°C (lane 7 and 8). In contrast, lower sensitivity was observed when the protein was treated with all the components required for the deglycosylation assay or some of them. Importantly, when ZIKV NS1 was treated only with denaturing buffer (lane 4), some effect on the detection of IgG antibodies was observed. It was stronger when the protein was treated with NP40 alone (lane 5) or in combination with other reagents (lane 1, 2, and 6). These results suggest an important role of the native conformation of ZIKV NS1, and possibly other flaviviruses for the detection of IgG antibodies by NS1-based ELISA test.

#### **3.4. Detection of IgM/IgG antibodies from sera samples of infected patients with TBEV, WNV, ZIKV, and DENV1-4**

I checked the presence of IgM/IgG antibodies in sera samples of RT-PCR confirmed patients infected by TBEV or WNV from endemic areas and infected patients by ZIKV or DENV1-4 from travelers. These sera samples were obtained from a collaboration with prof. Tatjana Avšič – Županc (university of Lubiana). The samples were also previously analyzed by commercial ELISA assays following the manufacturer's instructions. I analyzed the same samples by NS1-based ELISA following a similar protocol used before for the detection of antibodies from mice sera. In this case, human sera samples were diluted 1:20, and IgG antibodies were depleted previous IgM detection.

##### **3.4.1. Detection of IgM/IgG antibodies from a cohort of patients infected by TBEV**

To assess the sensitivity and specificity of the NS1-based ELISA test for the detection of IgM/IgG antibodies in infected patients by TBEV, I selected a total of 100 sera samples from TBEV cases tested positive by RT-PCR. 22 samples were collected during the first and second phase of the

infection (1-8 and 9-19 days after onset of symptoms, respectively), 34 sera samples were collected during the acute phase (from 20 days after onset of symptoms to 2 months), and the last 44 samples were obtained from patients in the convalescent phase of the infection (from 2 months after onset of the symptoms).

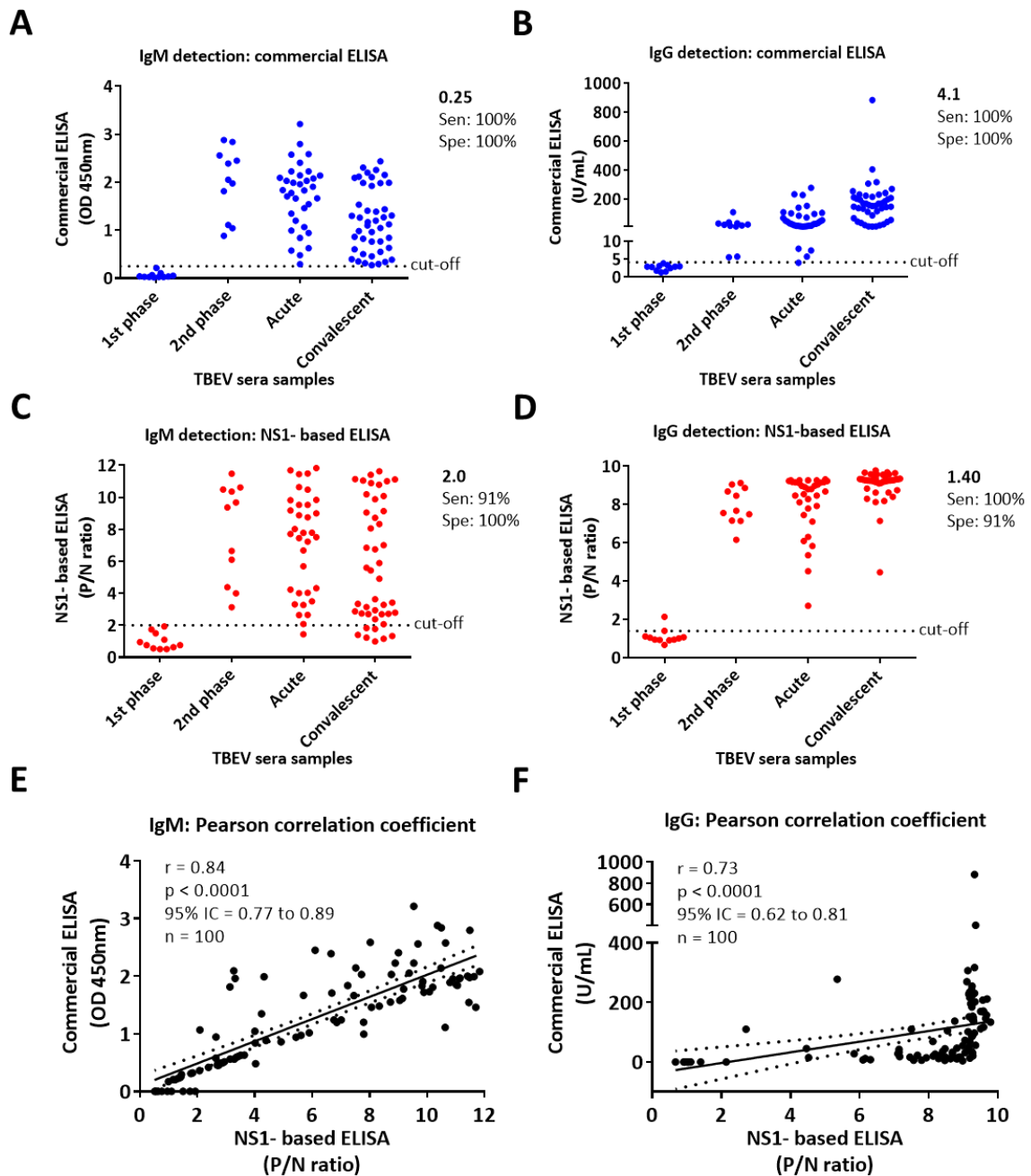
All the samples were tested for the presence of IgM/IgG antibodies by commercial ELISA (plates coated with inactivated TBEV antigen). It was done in collaboration with prof. Tatjana Avšič – Županc. I performed the NS1-based ELISA (plates coated with purified TBEV rNS1 antigens). The total number of positive samples obtained by both assays is shown in table 3.2. Cut-off values for commercial ELISA were calculated according to the manufacturer's instructions (IgM cut-off at 0.25 OD<sub>450</sub> and IgG at 4.1 U/mL); while, the calculation of the cut-off values for TBEV NS1-based ELISA assay was done based on the comparative receiver operating characteristic (ROC) curve analysis explained in the materials and methods (IgM cutoff fell at 2.0 and IgG at 1.4 P/N ratios).

TBEV samples (n = 100)	Days after onset of symptoms	RT-PCR positive samples	Commercial ELISA <sup>d</sup>		NS1-based ELISA <sup>e</sup>	
			IgM	IgG	IgM	IgG
<b>1<sup>st</sup> phase<sup>a</sup></b>	1 – 8	11	0	0	0	1
<b>2<sup>nd</sup> phase<sup>a</sup></b>	9 – 19	11	11	11	11	11
<b>Acute<sup>b</sup></b>	20 – 2 months	34	34	34	33	34
<b>Convalescent<sup>c</sup></b>	From 2 months	44	44	44	37	44

**Table 3.2. Number of positive samples from TBEV infected patients.** <sup>a</sup> A total of 11 samples were tested for each group, first (1<sup>st</sup>) and second (2<sup>nd</sup>) phase of TBEV infection, <sup>b</sup> A total of 34 samples were tested for the acute phase of TBEV infection, <sup>c</sup> A total of 44 samples were tested for the convalescent phase of TBEV infection, <sup>d</sup> plates were coated with inactivated TBEV antigen, Enzygnost; Simens GmbH, <sup>e</sup> Plates were coated with NS1 of TBEV.

Among the 100 samples analyzed by commercial ELISA, 11 samples from the first phase of infection were IgM/IgG negative, while all the samples from the second, acute and convalescent phases were IgM/IgG positive (Figure 3.14A and 3.14B, and Table 3.2). I obtained comparable results by NS1-based ELISA. 10 samples from the first phase of infection were IgM and IgG negative. Only one sample from the first phase turned IgG positive with a low P/N ratio. All the samples from the second phase were IgM/IgG positive. 33/34 and 37/44 samples from acute and convalescent phases, respectively, were IgM positive. All the samples from these two phases were also IgG positive (Figure 3.14C and 3.14D, and Table 3.2).





**Figure 3.14. Detection of IgM/IgG antibodies from TBEV infected individuals. A and B)** Detection of IgM (A) IgG (B) antibodies by commercial ELISA. IgM results are reported as OD<sub>450</sub>, while IgG results are reported as U/mL. Cut-off values for IgM (0.25) and IgG (4.1) were calculated according to the manufacturer's instructions. **C and D)** Detection of IgM (C) IgG (D) antibodies by rNS1-based ELISA. Optimal cut-off values of the P/N ratio (OD<sub>450</sub> of test specimen divided by the mean OD<sub>450</sub> of negative control specimens) were calculated based on the comparative receiver operating characteristic (ROC) curve analysis. Cut-off values for IgM and IgG fell at 2.0 and 1.40, respectively. **E and F)** Correlation between commercial and rNS1-based ELISA assays. The two-tailed Pearson's correlation value ( $r$ ) was calculated for IgM and IgG values. A  $P$  value of  $<0.0001$  rejected the null hypothesis that there exists no correlation between commercial and rNS1-based ELISA methods. 95% Interval Confidence (IC) value is also indicated and showed in dotted lines above and under the linear correlation. 100 sera samples ( $n$ ) from different phases of TBEV infection were included in the analysis. Each ELISA result includes an average of two biological replicates.

Based on 89 IgM/IgG positive samples (100% sensitivity) and 11 IgM/IgG negative samples (100% specificity) analyzed by commercial ELISA, I determined the sensitivity and specificity of the NS1-based ELISA with 95% confidence intervals (CI). The sensitivity was 91% and 100%, while the specificity was 100% and 91% for IgM (95% CI, 0.77 to 0.89) and IgG (95% CI, 0.62 to 0.81) detection, respectively (Figure 3.14E and 3.14F). Combined IgM/IgG sensitivity and specificity was 96% among the 100 TBEV-positive specimens tested.

### **3.4.2. Differential diagnosis of TBEV using rNS1 antigens**

The diagnosis of TBEV can be very complex due to the lack of a specific test. Importantly, seroconversion against TBEV can be obtained by natural infection or vaccination. However, some seroconverted patients, due to vaccination, can get naturally infected (Sendi, 2017). This group of vaccine break-through cases is seroconverted against the envelope antigen used in all the commercial ELISA tests available for the diagnosis of TBEV. It means that these tests can not differentiate between vaccinated or naturally infected people. The only way to do a serological test that differentiates these cases is by using the NS1 protein of TBEV that is not present in the vaccine.

Therefore, I compared the results obtained from 43 human sera samples analyzed for the presence of IgM/IgG antibodies by commercial ELISA (in collaboration with prof. Tatjana Avšič – Županc) and NS1-based ELISA assays (Figure 3.15A and 3.15B, and Table 3.3). Of the 43 specimens, 10 healthy and 3 sera samples of individuals from endemic areas who got the YFV vaccine (live attenuated 17D vaccine) were included as controls. All 13 sera samples were from TBEV non-endemic regions. The other 30 sera samples include 10 individuals who got the TBEV vaccine (a suspension of purified TBE inactivated virus), 10 patients with TBEV acute-phase infection, and 10 sera from vaccine breakthrough (VBT) patients.

The total number of positive samples analyzed by commercial and NS1-based ELISA assays are shown in table 3.3. When the samples were analyzed using the commercial ELISA, all samples from YFV and TBEV vaccinated groups were IgM negative, while 10/10 and 7/10 sera samples from TBEV acute-phase infection or VBT, respectively, were IgM positive. Analysis using the commercial ELISA test also showed that all sera samples were IgG positive, including 3 sera samples from the YFV vaccinated group (Table 3.3). However, only sera samples from TBEV acute-phase infection and VBT patients were all IgM and IgG positive when they were analyzed by NS1-based ELISA. The rest of the samples analyzed by NS1-based ELISA were IgM and IgG

negative (Figure 3.15A and 3.15B, and Table 3.3). This data indicates the specificity and the potential of TBEV NS1 to make a differential diagnosis of TBEV.

TBEV sera samples (n = 43)	Commercial ELISA <sup>a</sup>		rNS1-based ELISA <sup>b</sup>	
	IgM	IgG	IgM	IgG
Healthy	NA	NA	0/10	0/10
YFV vaccinated	0/3	3/3	0/3	0/3
TBEV vaccinated	0/10	10/10	0/10	0/10
TBEV acute-phase	10/10	10/10	10/10	10/10
VBT	7/10	10/10	10/10	10/10

Table 3.3. Number of TBEV positive IgM/IgG samples out of the total number of samples analyzed by commercial ELISA and NS1-based ELISA assays. VBT: vaccine breakthrough, NA: not-analyzed, <sup>a</sup> IgM/IgG antibodies were detected by using a commercial kit according to the manufacturer's instructions for TBEV (plates were coated with inactivated TBEV E antigen, <sup>b</sup> IgM/IgG antibodies were detected by NS1-based ELISA coating the plates with the rNS1 protein of TBEV.

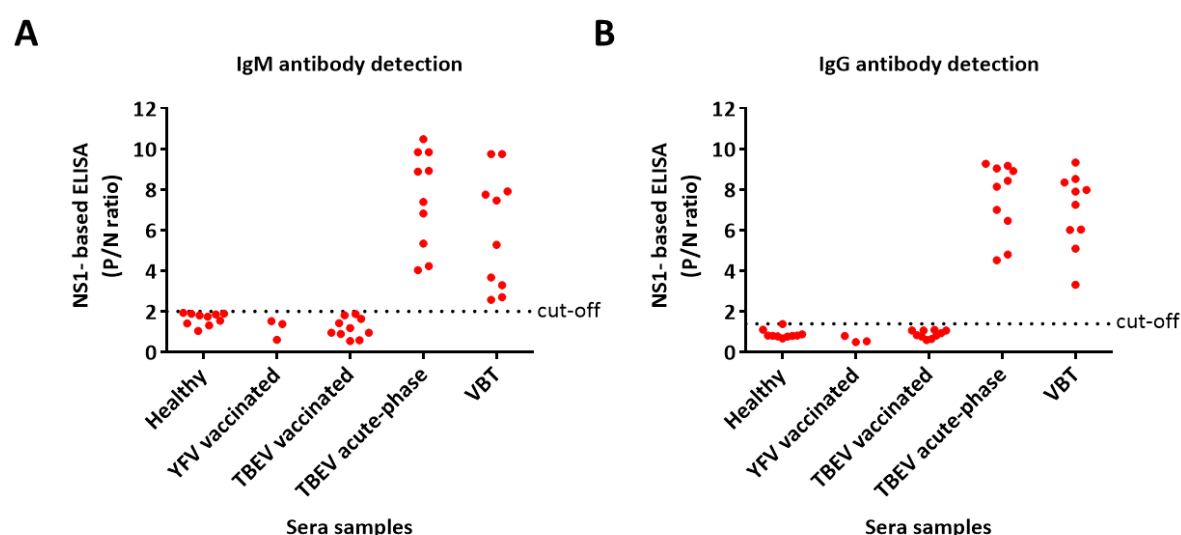


Figure 3.15. Differential serological diagnosis of TBEV. A and B) Detection of IgM (A) IgG (B) antibodies by NS1-based ELISA using sera samples from healthy individuals, YFV, or TBEV vaccinated individuals, individuals with acute TBEV infection, and vaccine breakthrough (VBT) group. Optimal cut-off values of P/N ratios were calculated based on ROC curve analysis. Cut-off values for IgM and IgG fell at 2.0 and 1.40, respectively. Each ELISA result includes an average of two biological replicates.

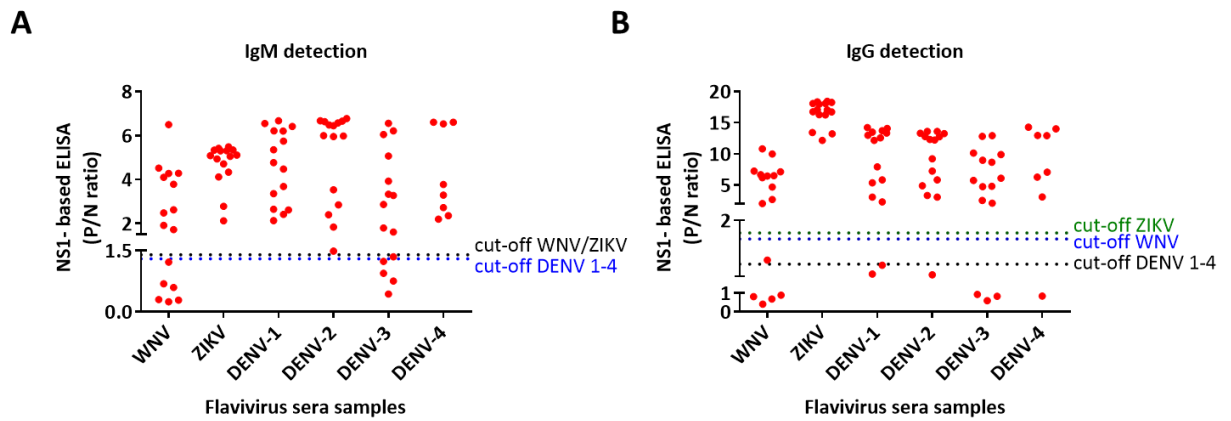
### 3.4.3. Detection of IgM/IgG antibodies from a cohort of patients infected by WNV, ZIKV or DENV 1-4

I selected a cohort of 84 specimens from RT-PCR confirmed patients with flavivirus infection (including travelers firstly exposed to DENV infection). Among the specimens, 16 samples were positive for WNV, 15 for either ZIKV or DENV1, 2 or 3, and 8 for DENV4. The positivity of a P/N ratio results obtained by NS1-based ELISA was determined according to the ROC cut-off value described in the materials and methods. The optimal cut-off value for IgM detection of WNV and ZIKV fell at 1.4, while for IgG detection, it fell at 1.7 and 1.8 P/N ratios, respectively. For the detection of IgM and IgG antibodies against all DENV serotypes, both cut-off values fell at 1.3 P/N ratio (Table 3.4).

	<b>rNS1 antigens</b>					
	<b>WNV</b>	<b>ZIKV</b>	<b>DENV-1</b>	<b>DENV-2</b>	<b>DENV-3</b>	<b>DENV-4</b>
<b>IgM</b>	1.4	1.4	1.3	1.3	1.3	1.3
<b>IgG</b>	1.7	1.8	1.3	1.3	1.3	1.3

**Table 3.4. Optimal cut-off values of the P/N ratio for IgM/IgG antibody detection from sera of positive patients with flavivirus infection by NS1-based ELISA.** Cut-off values were determined for each antigen based on 13 negative sera samples from endemic regions. rNS1: recombinant non-structural protein 1, WNV: west nile virus, ZIKV: zika virus, DENV1-4: dengue virus serotype 1, serotype 2, serotype 3, and serotype 4.

Each sera sample was tested for the presence of IgM/IgG antibodies by commercial ELISA (in collaboration with prof. Tatjana Avšič – Županc), and the number of positives samples are shown in table 3.5. The same samples were also analyzed by NS1-based ELISA (Figure 3.16A and 3.16B, Table 3.5).



**Figure 3.16. Detection of IgM/IgG antibodies from WNV, ZIKV, and DENV 1-4 infected individuals. A and B)** Detection of IgM (A) IgG (B) antibodies by NS1-based ELISA. Each group of samples from RT-PCR-confirmed patients were screened for the presence of IgM/IgG antibodies by NS1-based ELISA tests using purified rNS1 antigens. Optimal cut-off values of P/N ratios were calculated based on ROC curve analysis. Each cut-off was selected based on the P/N ratio value, which gave 100% sensitivity and specificity. Each ELISA result includes an average of two biological replicates.

12/16 IgM and 9/16 IgG positive WNV sera samples were positive by a commercial ELISA (plates coated with recombinant WNV E antigen, Focus Diagnostics). Whereas, 10/16 IgM and 12/16 IgG positives were obtained by WNV NS1-based ELISA. 11/15 IgM and 14/15 IgG ZIKV positive sera samples were detected by a commercial ELISA (plates coated with ZIKV recombinant NS1 antigen, Euroimmune; Labordiagnostika AG), while all fifteen ZIKV sera samples were IgM/IgG positive when they were tested by the ZIKV NS1-based ELISA assay (Figure 3.16A and 3.16B, Table 3.5).

Specific DENV serotypes samples were analyzed for the presence of IgM/IgG antibodies using the same commercial ELISA assay (plates coated with DENV type 2 E antigen, NovaTec, immunodiagnostic GmbH). 10/15 IgM and 15/15 IgG for DENV1, 11/15 IgM, and 15/15 IgG for DENV2 and 10/15 IgM and 13/15 IgG for DENV3 resulted positive, while 3/8 IgM and 8/8 IgG resulted positive for DENV4. In comparison, using the NS1-based ELISA (plates coated with serotype-specific rNS1 antigen), all 15 sera samples from DENV1 and DENV2, and 8 sera from DENV4 infected patients were positive for IgM detection, and 13/15, 14/15, and 7/15 were IgG positive, respectively. Among 8 samples from DENV4 infected patients, only one sample was IgG negative, while all the others were IgM and IgG positive (Figure 3.16A and 3.16B, Table 3.5).

Sera samples (n = 84)	RT-PCR-positive samples	Commercial ELISA <sup>a</sup>		NS1-based ELISA <sup>b</sup>	
		IgM	IgG	IgM	IgG
<b>WNV</b>	16	12	9	10	12
<b>ZIKV</b>	15	11	14	15	15
<b>DENV-1</b>	15	10	15	15	13
<b>DENV-2</b>	15	11	15	15	14
<b>DENV-3</b>	15	10	13	11	12
<b>DENV-4</b>	8	3	8	8	7

**Table 3.5. Number of positive IgM/IgG samples from flavivirus infected patients.** <sup>a</sup> IgM/IgG antibodies were detected by using commercial kits according to the manufacturer's instructions for WNV (plates coated with recombinant WNV E antigen, Focus Diagnostics), ZIKV (plates coated with ZIKV recombinant NS1 antigen, Euroimmune; Labordiagnostika AG) and, DENV1-4 (plates coated with DENV type 2 E antigen, NovaTec, immunodiagnostic GmbH). <sup>b</sup> IgM/IgG antibodies were detected using purified rNS1 proteins of WNV, ZIKV, and all DENV serotypes.

### 3.4. Molecular detection and seroprevalence studies of flavivirus in Italy and Nigeria

#### 3.4.1. USUV in blood donors from the North-Eastern region in Italy

Usutu virus (USUV) is an emerging flavivirus in Europe circulating in migratory birds and transmitted to humans by mosquitoes in 2001 (Ashraf et al. 2015; Cheng et al. 2018). Italy is endemic for west Nile virus, and cases of USUV transmission have also been reported, some with neurological complications (Pecorari et al. 2009). However, systematic screening for USUV is only starting to be implemented. Therefore, surveillance of USUV circulation, as well as monitoring of USUV evolution, will be crucial to try to prevent future outbreaks both in Europe and in other countries currently naïve to the infection such as the Americas.

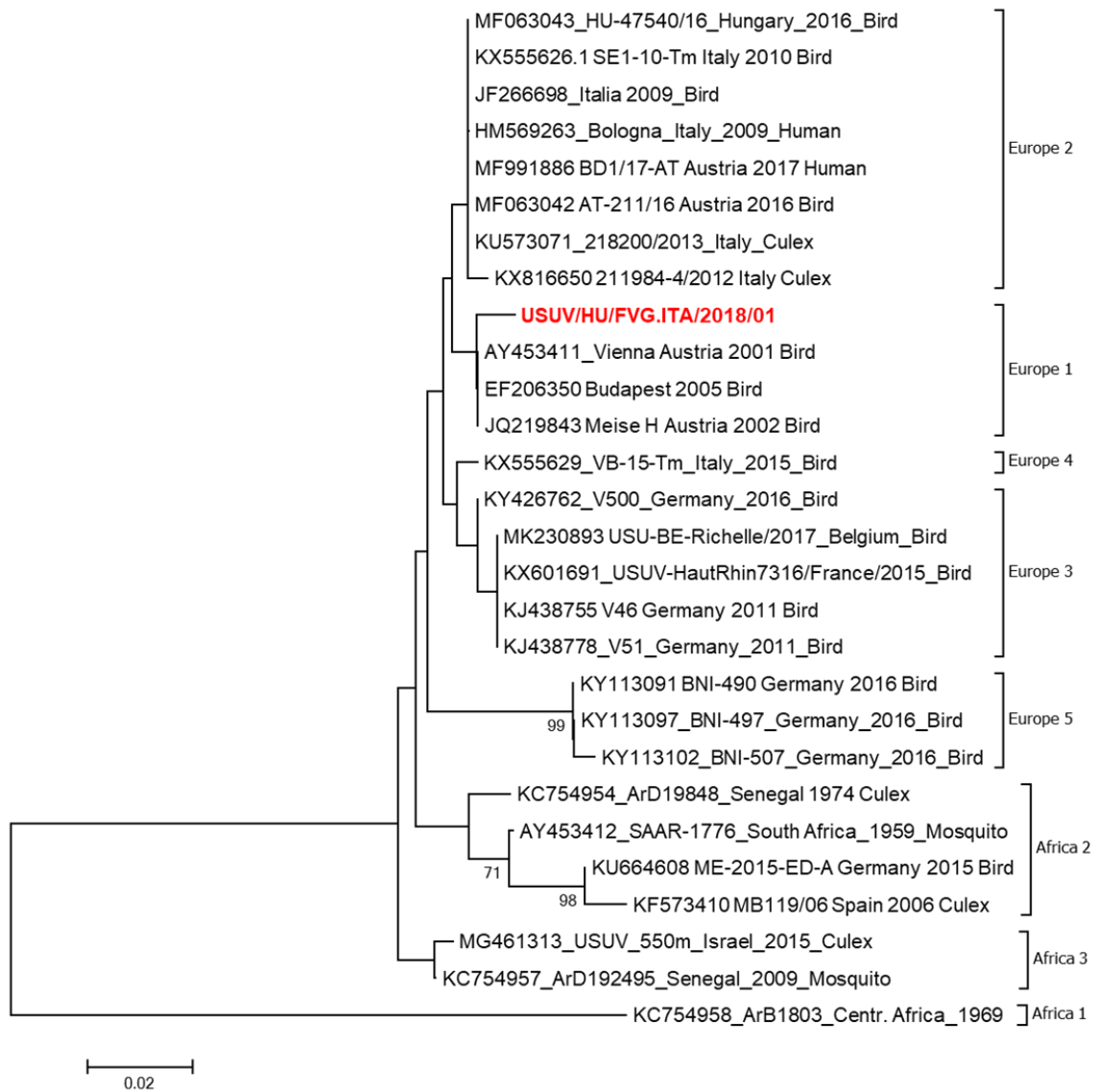
Here I report the results of the first isolation of USUV in the North-Eastern region in Italy, the IgM/IgG serological evidence of two USUV cases, and a retrospective study for the detection of antibodies in blood donors from the North-Eastern region in Italy. The results presented in this section indicate not only the current but also the past exposure to USUV in the region.

#### **3.4.1.1. First isolation and identification of USUV in the North-Eastern region of Italy**

In collaboration with the Regional Reference Laboratory for Arbovirus Diagnosis (RRLAD) in Trieste-Italy, we isolated for the first time USUV from a serum sample of a blood donor (patient 1). Blood donations in the Friuli Venezia Giulia Region, an area endemic for WNV, are routinely screened by the Cobas® WNV nucleic acid test, and positive results are sent for confirmation to the RRLAD in Trieste. In August 2018, an asymptomatic blood donor positive in the initial screening test could not be confirmed for WNV RNA. However, the sample turned positive for USUV RNA by the real-time RT-PCR amplification protocol of Cavrini et al. (Cavrini et al. 2011). The infectious virus was rescued from Vero cells inoculated with the human serum and a sequence of a 659 bp conserved region of NS5 was obtained (accession number MN509808). This novel isolate, named USUV/HU/FVG.ITA/2018/01 was shown to cluster with USUV strains from lineage Europe 01 isolated in Austria, Hungary, and Germany (Figure 3.17). This is the first documented case of USUV transmission to humans in North-Eastern Italy and indicative of USUV circulation in the area.

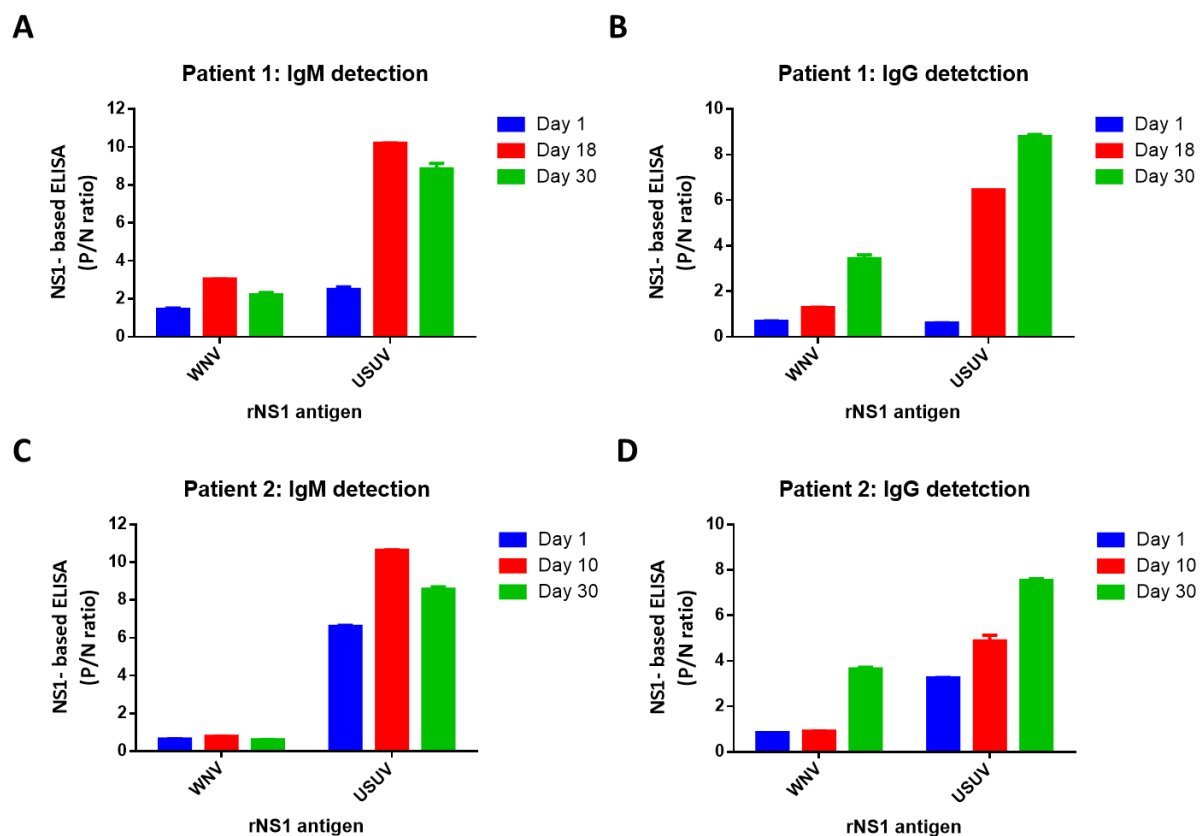
#### **3.4.1.2. First serological evidence of USUV in the North-Eastern region of Italy**

From the same asymptomatic blood donor (patient 1) positive for USUV RNA by real-time RT-PCR in the first sample collection (1 day from blood donation), I received three different sera samples (1, 18, and 30 days from blood donation) to check the presence of IgM and IgG antibodies by USUV NS1-based ELISA. Since there is some cross-reactivity between USUV and WNV antibodies, I checked the presence of IgM/IgG antibodies against rNS1 of both viruses (Figure 3.18A and 3.18B). Low IgM/IgG P/N ratios were detected when the samples were reacted against the rNS1 of WNV; however, when the same samples were tested on the rNS1 protein of USUV, the seroconversion characteristic of a new infection confirmed the detection of the first USUV case circulating in the North-Eastern region in Italy. Comparable seroconversion to USUV was observed in follow-up samples (1, 10, and 30 days from blood donation) from a different blood donor (patient 2) (Figure 3.18C and 3.18D).



**Figure 3.17. Phylogenetic tree of USUV lineages, including the isolate from an asymptomatic blood donor.** Neighbor-Joining evolutionary history calculated with MEGA X software from USUV sequences following BLAST analysis and alignment of the USUV\_HU\_FVG.ITA\_2018\_01 sequence (shown in red). Clusters of closely related sequences are shown for each USUV lineage, with an indication of host and geographical origin.



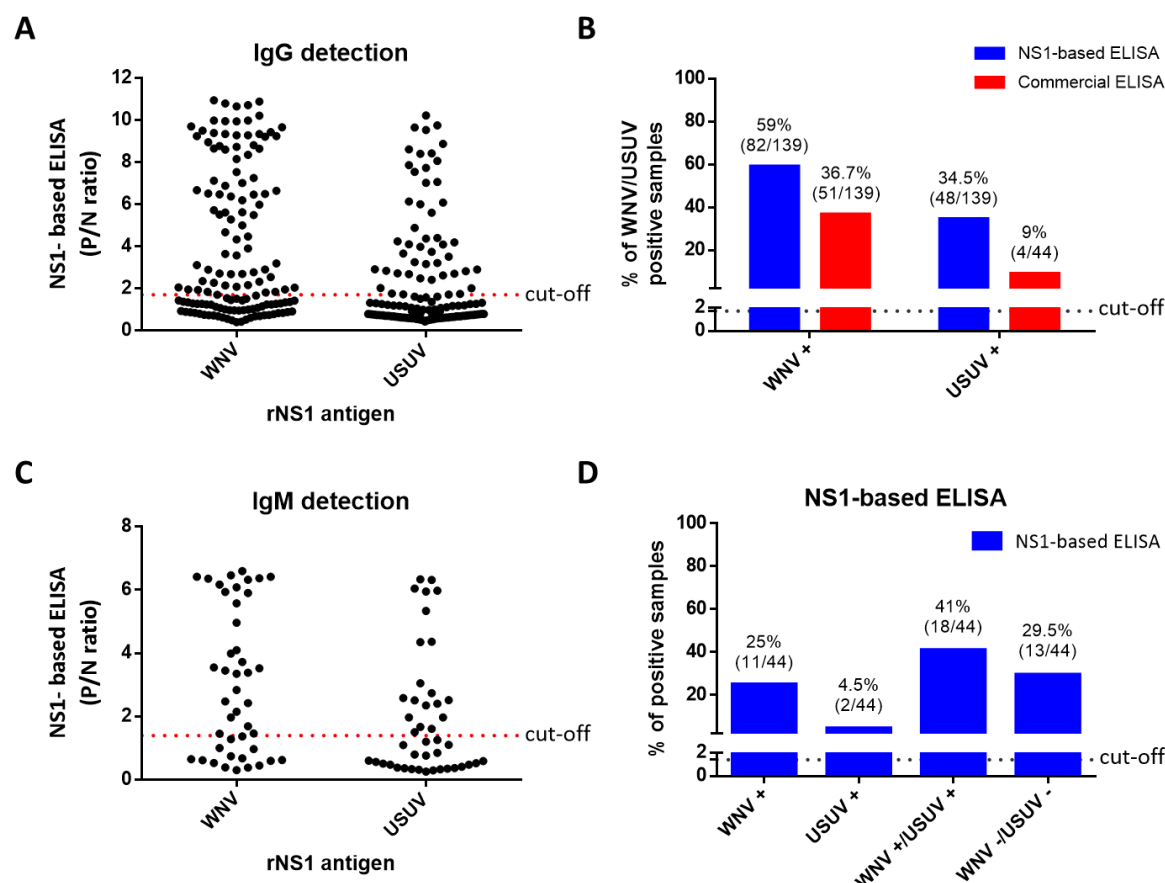


**Figure 3.18. Serological detection of USUV in the North-Eastern region of Italy. A – D)** Detection of IgM and IgG antibodies by NS1-based ELISA. Plates were coated with the recombinant NS1 antigen of USUV and WNV. Three different sera samples from two asymptomatic patients were tested: A and B) samples from patient 1 were collected 1, 18, and 30 days from blood donation, while samples from patient 2 (C and D) were collected 1, 10, and 30 days from blood donation. Each ELISA result includes an average of two biological replicates. Cut off values are not indicated due to the low number of certified samples positive for USUV.

#### 3.4.1.3. Retrospective analysis of the seroprevalence of USUV in blood donors

A total of 139 sera samples from blood donors that were sent for confirmation of suspect WNV infection in 2018 were used to detect IgG antibodies to USUV and WNV by the novel NS1-based ELISA established in this work (Figure 3.19). The same number of samples were also tested for the detection of IgG antibodies to WNV by the Focus Diagnostics commercial ELISA assay (plates coated with recombinant WNV envelope antigen). Since the systematic screening for USUV is only starting to be implemented, only 44 out of 139 sera samples were analyzed by the commercial Euroimmun IgG ELISA (plates coated with recombinant USUV envelope antigen). The same 44 samples were also tested for the presence of IgM and IgG antibodies by NS1-based ELISA. The detection of IgM antibodies was not performed by commercial ELISA given the lack of an assay for the purpose.

As expected, I found high seroprevalence of IgG antibodies to WNV in blood donors of North-Eastern Italy (Figure 3.19A and 3.19B). 59% (82/139) and 36.7% (51/139) of the total number of samples were IgG positive by WNV NS1-based ELISA and commercial ELISA assays, respectively. Surprisingly, I also found a high percentage of IgG positive sera samples to USUV (Figure 3.19A and 3.19B). 34.5% (48/139) of the samples turned IgG positive when they were analyzed by the NS1-based ELISA coating the plates with rNS1 of USUV, while only 9% (4/44) of sera samples were positive for USUV using the commercial Euroimmun IgG ELISA assay.



**Figure 3.19. Detection of IgM and IgG antibodies from blood donors.** **A)** Detection of IgG antibodies from 139 sera samples of blood donors by NS1-based ELISA assay. An optimal cut-off value of the P/N ratio for IgG fell at 1.7. **B)** Comparison of the percentage of IgG positive sera samples tested by NS1-based ELISA and commercial assays. The number of positive samples out of the total number of samples analyzed by each assay is shown in parenthesis. **C)** Detection of IgM antibodies from 44 sera samples of blood donors by NS1-based ELISA assay. An optimal cut-off value of the P/N ratio for IgM fell at 1.4. **D)** Percentages of positive (+) samples for only WNV, only USUV, both viruses (WNV/USUV), and negative (-) samples for both viruses. Each ELISA result includes an average of two biological replicates.

Among the 44 samples tested for the presence of IgM antibodies against USUV and WNV by NS1-based ELISA, 66% (29/44) and 45.40% (20/44) were positive for WNV and USUV,

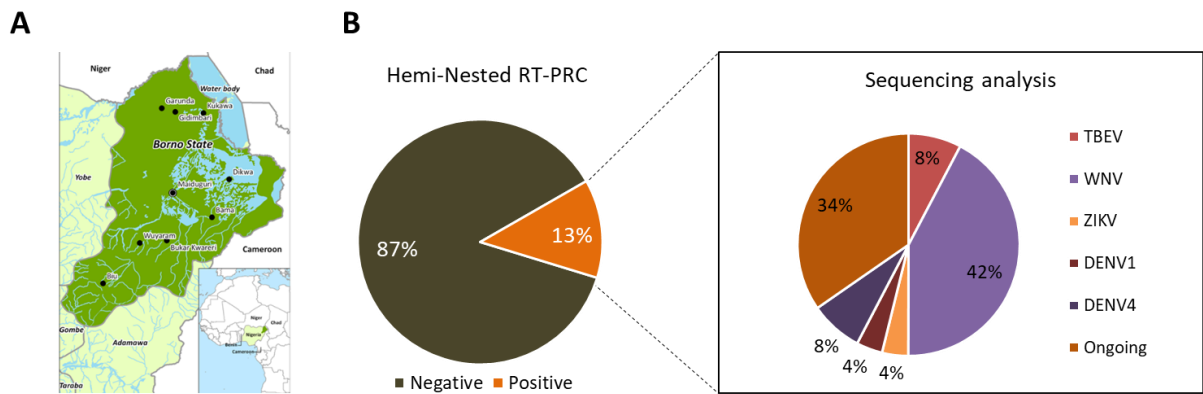
respectively (Figure 3.19C). In this group of samples, 25% (11/44), 4.5% (2/44), and 41% (18/44) were IgM positive only for WNV, USUV, or both viruses, respectively. 29.50% (13/44) of the samples were negative for both viruses (Figure 3.19D). These results indicate the high specificity of the NS1-based ELISA to differentiate WNV and USUV infections.

According to the number of samples analyzed by each assay, the novel NS1-based ELISA showed higher sensitivity for the detection of IgG antibodies compared to the commercial ELISA. The novel NS1-based ELISA assay also showed high specificity. Including the two IgM/IgG USUV positive cases mentioned above, I found 5 sera samples that were only IgG positive to rNS1 of USUV confirming the past exposure to the virus, while commercial ELISA only allowed the identification of one possible case of USUV. It remarks the importance of using the rNS1 protein of USUV for differential diagnosis, especially in regions where WNV is endemic.

### **3.4.2. Molecular detection and seroprevalence of flavivirus in Borno State, north-eastern Nigeria**

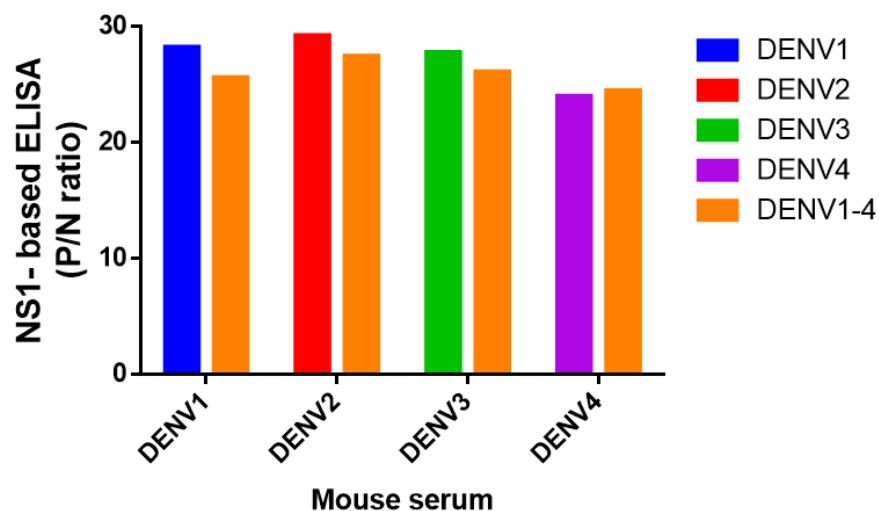
Lack of accessible, affordable, and appropriate diagnostic facilities in Africa impedes systematic surveillance leading to under-reporting and under-estimation of arboviral infections. In addition, in many African countries, including Nigeria, flavivirus diagnosis can easily be mistaken with other febrile illnesses that are presumptively diagnosed as malaria or typhoid (Baba et al. 2009; Onoja et al., 2016). Therefore, the establishment of a diagnostic laboratory for arboviral infections in Nigeria is crucial to active surveillance towards the prevention and control of these infections. To this end, in collaboration with prof. Marycelin Baba from the University of Maiduguri Teaching Hospital (UMTH) in Nigeria, we tested 200 sera samples from patients exhibiting febrile illness who visited the UMTH in Borno State (Figure 3.20A) for medical attention between March and April 2018.

All the samples were tested for the presence of viral RNA following the protocol of Scaramozzino et al. (Scaramozzino et al. 2001), which utilizes a pan flavivirus primer set for hemi-nested RT PCR. Among the 200 samples, twenty-six samples (13%) sera samples were positive for flaviviral RNA (by hemi-nested RT PCR) and ongoing sequence analysis assigned 11 (42%) to WNV, 1 (4%) to DENV2 and ZIKV, and 2 (8%) to DENV4 and TBEV western isolate Hyper (Figure 3.20B).



**Figure 3.20. Detection of flavivirus in Borno State, north-eastern Nigeria.** **A)** map of north-eastern Nigeria where the 200 sera samples were collected between March and April 2018. **B)** Percentage of positive/negative human sera samples by hemi-nested RT-PCR (left graph). Preliminary sequencing results from the positive hemi-nested RT-PCR results are also shown (right graph). Ongoing corresponds to positive samples by hemi-nested RT-PCR, in which the sequencing is being processed.

To understand the flavivirus seroprevalence in north-eastern Nigeria, the 200 sera samples were also analyzed for the presence of IgG antibodies against TBEV, WNV, ZIKV, USUV, and all four DENV serotypes by NS1-based ELISA assay established in this work.

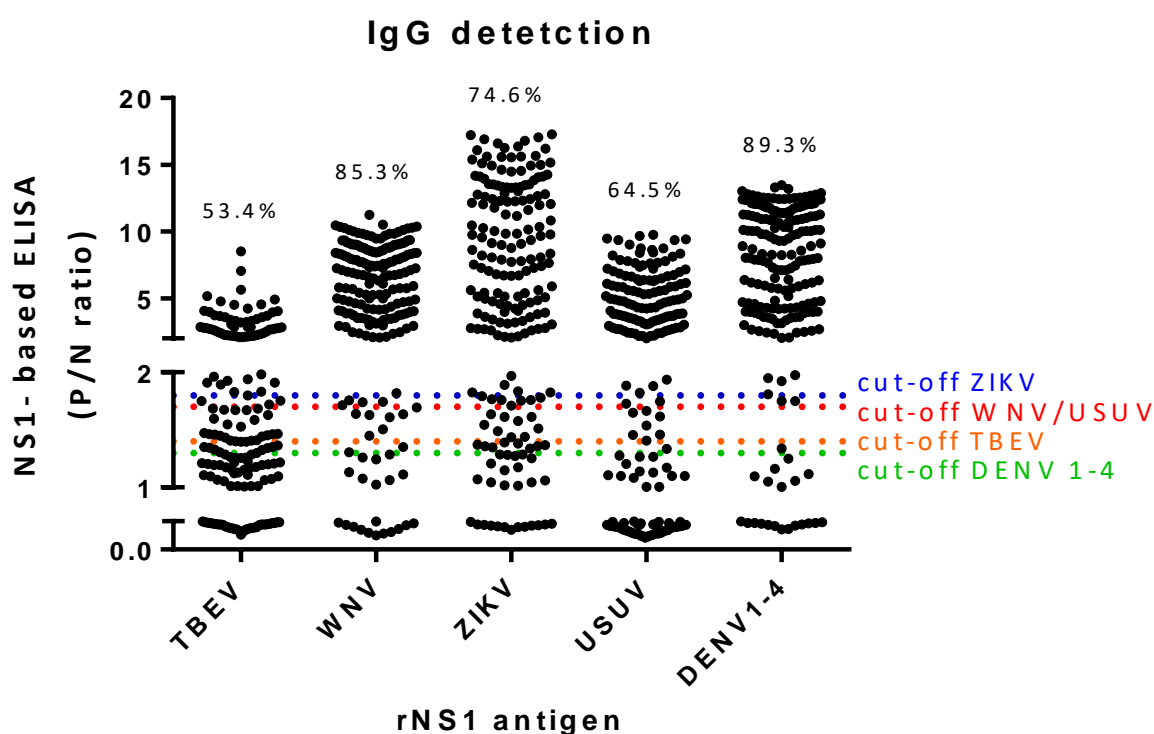


**Figure 3.21. Detection of IgG antibodies from DENV1-4 immunized mice.** Sera from immunized mice with DENV1, 2, 3, or 4 were used for IgG detection by NS1-based ELISA assay. Plates were coated with rNS1 of individual DENV serotypes, or an equimolar combination of all four antigens (DENV1-4). The results are shown as the P/N ratio and include an average of two biological replicates.

Since some cross-reactivity between IgG antibodies developed against rNS1 proteins of all four DENV serotypes was observed previously (Figure 3.11), the analysis for the detection of IgG antibodies against DENV was performed by an NS1-based ELISA where plates were coated with

a mix of all recombinant NS1 antigens at an equimolar concentration (1.25µg/ml). The P/N ratio values for each DENV serotype were comparable when the plates were coated with an individual serotype or an equimolar combination of all four antigens. It is shown using sera from immunized mice with individual DENV serotypes (Figure 3.21).

P/N ratio values above the cut-off were obtained for all flaviviruses tested (Figure 3.22). High percentage of IgG positive samples were detected for DENV1-4 (89.3%) and WNV (85.3%), followed by ZIKV (74.6%), USUV (64.5%) and TBEV (53.4%). Some of the samples were positive only for a single virus. Together, 93.5% of the sera samples had IgG antibodies for at least one flavivirus, while only 6.5% of the patients were negative for all the flaviviruses tested. These results identify the North-Eastern part of Nigeria with a high prevalence rate of flaviviruses. Additionally, these data support the use of recombinant NS1 protein as a valuable option for the detection of flavivirus antibodies with reduced cross-reactivity and high sensitivity, as well as, the implementation of the NS1-based ELISA assay for surveillance studies.



**Figure 3.22. Seroprevalence of IgG antibodies to flavivirus in Borno-State, Nigeria.** Detection of IgG antibodies by NS1-based ELISA assay from febrile illness patients. The percentage of positive samples is indicated for each virus. Optimal cut-off values of P/N ratios calculated based on ROC curve analysis are indicated for each virus. Each ELISA result includes an average of two biological replicates.

## DISCUSSION

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#### 4. DISCUSSION

Cross-reactivity between different flaviviruses has been the main drawback in the serological assays available for the diagnosis of flavivirus infections (Koraka et al. 2002; Mansfield et al., 2011). Many of them are based on the detection of antibodies against envelope protein that poorly allow the differentiation among flavivirus infections (Priyamvada et al. 2016). Much effort has been dedicated generating recombinant envelope proteins or virus-like particles containing mutations in the fusion loop domain to reduce the detection of cross-reacting antibodies (Chiou et al. 2008; Rockstroh et al. 2018; Rizzo et al. 2019; Rockstroh et al. 2015, 2017); however, the problem remains unsolved.

Several reports have proposed the use of non-structural proteins as antigens for specific diagnosis of flaviviruses. The presence of IgM and IgG antibodies against NS1, NS3, and NS5 proteins have been detected in acute-phase sera samples of DENV infected patients (Valdes et al. 2000; Alvarez-Rodriguez et al. 2012; Narayan et al. 2016; Tyson, Tsai, Tsai, Ma, et al. 2019). It was demonstrated by western blot analysis using viral proteins concentrated from infected Vero cells. More recently, the detection of antibodies by NS5-based ELISA has proposed NS5 protein as an antigen for the detection of st. louis encephalitis, west wile and dengue virus infections (Simari et al. 2019; Wong et al. 2003; Zhang et al. 2019); however, the NS5 protein used in these studies, which was purified from inclusion bodies in *E. coli*, did not preserve its native conformation and was not glycosylated.

More robust evidence for the detection of IgM and IgG antibodies against different flaviviruses has been reported using NS1 protein produced in mammalian cells as an antigen for ELISA assays (Cleton et al. 2015; Nascimento, George, et al. 2018; Nascimento, Huleatt, et al. 2018; Steinhagen et al. 2016; Tyson, Tsai, et al. 2019). However, some contradictory results in the detection of IgM/IgG antibodies from patients of flavivirus-endemic regions put in question the accuracy of the current available NS1-based assays based on commercial sources of antigen (L'Huillier et al. 2017).

To address the usefulness of NS1-based assays for the detection of flavivirus antibodies, I optimized the conditions to produce and purified recombinant NS1 proteins of different flavivirus in their native conformation from the supernatant of transient transfected mammalian cells. I used them to develop a novel NS1-based ELISA for the detection of IgM and IgG antibodies. The antigenicity and the specificity of the purified antigens were analyzed using

sera samples from immunized mice. The NS1-based ELISA was validated for the detection of IgM/IgG antibodies from well-characterized human sera samples by comparing the results with commercially available ELISA assays. The optimized NS1-based ELISA was used to assess the seroprevalence of USUV in the North-Easter region in Italy, as well as the seroprevalence of flaviviruses in the North-Easter region in Nigeria.

The data presented in the figures and results chapter support the usefulness of the NS1-based ELISA in flavivirus serology, which will be discussed in this section.

#### **4.1. Production of recombinant NS1 proteins**

Different strategies for the expression and purification of flavivirus recombinant NS1 (rNS1) proteins have been reported. NS1 protein of Japanese encephalitis, St. Louis Encephalitis, dengue virus serotype 1-3 and west Nile virus has been purified from bacteria (Athmaram et al. 2012; Das et al. 2009; Dhanze et al. 2019; Lorch et al. 2019; Sankar et al. 2013) or Sf21 cells in case of DENV4 (Gelanew and Hunsperger 2018); while protocols to produce ZIKV NS1 from *Drosophila* S2 stable clone or DENV2 NS1 from *Pichia pastoris* yeast cells have also been established (Allonso et al. 2019; Tsai et al. 2017; Tyson, Tsai, Tsai, Brites, et al. 2019; Tyson, Tsai, Tsai, Ma, et al. 2019). All these strategies have been optimized in systems that are prone to problems of protein stability and/or lack the proper folding and post-translational modifications. For this reason, I optimized the conditions to express rNS1 proteins of TBEV, WNV, ZIKV, USUV, and all four DENV serotypes in transiently transfected HEK-293T cells.

Apart from commercially available flavivirus NS1 antigens produced in mammalian cells (The Native Antigen Company), there are not reports about the development of strategies to purify the NS1 protein of TBEV in any system. Only NS1 of USUV has been produced in HEK-293 cells and purified from the culture media of a stable clone (Cleton et al. 2015).

Expression of recombinant NS1 proteins in mammalian cells is essential to preserve the pattern of posttranslational modifications, stability, and folding into a functional structure (Blitvich et al. 2001; Dumont et al. 2016; Lin et al. 2015; Pryor and Wright 1994; Hu et al. 2018). However, I observed that low expression level of recombinant proteins' expression is a problem shared among NS1 proteins. To overcome this, I optimized the expression of rNS1 proteins in HEK-293T cells by treating them with sodium butyrate. This compound is a histone deacetylase inhibitor that like valproic acid, is very efficient in enhancing recombinant protein expression



in mammalian cells (Backliwal et al. 2008; Fan et al. 2005; Jeon and Lee, 2007). This compound induces histone hyper-acetylation that results in more flexible chromatin by reducing DNA–histone interactions and thereby increase transcription levels. As compared to mild hypothermia and overexpression of cell cycle kinases, the addition of histone deacetylase inhibitors could also induce growth arrest without adverse effects on cell growth and cell viability (Kiszel et al. 2015; Jeon and Lee, 2007; Sunley and Butler 2010; Wulhfard 2009). Although I did not perform any experiment to evaluate the effects of the treatment of HEK-293T cells with sodium butyrate, I did not observe any adverse effect on treated cells. On the contrary, I saw a significant increase in the expression and secretion of recombinant NS1 proteins assessed by western blot analysis on treated cells compared to untreated.

High yield NS1 purification of TBEV, WNV, ZIKV, USUV, and all four DENV serotypes was achieved from the supernatant of transiently transfected HEK-293T cells. From twenty 150 mm plates, I purified approximately 1 mg/mL of recombinant protein. In line with previous reports showing the ability of sodium butyrate to increase the expression of recombinant proteins, these findings suggest that supplementation of culture media with this compound is an efficient treatment to enhance the expression of NS1 proteins in HEK-293T cells.

#### **4.2. Conformational and glycosylation status of purified NS1 proteins**

The flavivirus genome is translated directly from the RNA as a single polyprotein comprising three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Perera and Kuhn 2008). The monomeric form of NS1 is cleaved from the preceding envelope protein by endoplasmic reticulum (ER) resident host signal peptidase (Nowak et al. 1989), while the NS1-NS2A cleavage is mediated by a host membrane-bound reticulum-resident protease (Falgout and Markoff 1995). The monomeric NS1 quickly dimerizes in the lumen of the endoplasmic reticulum (ER); at the same time, the addition of N-glycan starts in the ER, followed by glycan trimming in the Golgi apparatus (Flamand et al. 1999; Pryor and Wright 1994). Finally, NS1 glycoprotein is secreted as a lipid-associated barrel-shaped hexamer (Gutsche et al. 2011). The hexamer formation mechanism remains unclear, but it has been suggested to occur immediately after dimerization into the ER or possibly during the passage of NS1 through the Golgi secretory pathway (Muller and Young 2013).

In line with previous reports (Akey et al. 2014; Flamand et al. 1999), purified NS1 proteins from mammalian cells were characterized by the expected oligomeric state. Under native

conditions, labile hexameric oligomers and heat-sensitive dimeric oligomers were identified. These results confirm initial evidence related to the dimeric stability of NS1 proteins (Winkler et al. 1989). Interestingly, only TBEV NS1 protein was detected as a stable hexamer when the analysis was performed in the presence of reducing agents and without denaturing the protein. It could suggest that TBEV NS1 protein secreted from HEK-293T cells is composed of hexameric oligomers. The major difference between TBEV NS1 respect to other flaviviruses is the glycosylation profile. A unique conserved glycosylation site characterizes this protein at asparagine 207 (N-207) with two other possible glycosylation sites (N-85 and N-223) that are not conserved between flaviviruses (Mandl, Heinz, and Kunz 2004). More structural and conformational assays are needed to understand the role of the glycosylation profile in the stability of TBEV NS1.

Native conformation, together with proper glycosylation of NS1, has been demonstrated to be essential for hexamer stability, cell-surface expression, and secretion of NS1 (Somnuk et al. 2011). Additional studies have also shown that glycosylation mutants of NS1 proteins affect the production of viable viruses and cause reduced interaction of circulating NS1 with glycosaminoglycans, heparin sulfate, and chondroitin sulfate E present on the cell surface (Avirutnan et al. 2007). Most importantly the presence of glycans and some linear epitopes in the outer face of the hexameric form of NS1 have been demonstrated by computational analysis suggesting the importance of specific epitopes for the detection of antibodies against NS1 protein (Akey et al. 2014; Allonso et al. 2011; Jones et al. 2017).

I analyzed the presence of these posttranslational modifications in purified NS1 proteins from the supernatant of transfected HEK-293T cells by comparing the susceptibility of recombinant proteins to digestion with PNGase F and Endo Hf. Western blot analysis showed a similar effect of the NS1 treatment with one or both endoglycosidases. In all cases, the digestion with PNGase F was observed to induce a shift in-band migration concerning the untreated protein. A comparable shift, but slightly less evident, was observed when NS1 proteins were treated with Endo Hf. These results indicate the presence of N-linked high-mannose or hybrid oligosaccharide side chains characteristic of NS1 proteins produced in mammalian cells (Flamand et al. 1999; Mason 1989; Pryor and Wright 1994). Only WNV NS1 protein showed a different deglycosylation pattern when it was digested with endo Hf. The band migration was comparable to proteins treated with PNGase F, and a small fraction did not show a shift of band migration. The differential deglycosylation profile of WNV NS1 has already been

explained due to the inaccessibility of the endoglycosidase to the cutting site (Flamand et al. 1999), suggesting the presence of complex sugars that are resistant to the treatment with Endo Hf. The pattern of WNV NS1 digested with Endo Hf could be explained as the result of combining the efficient heterologous sec leader peptide and the RQ10NK mutation that confers high secretion efficiency (Youn et al., 2010). Further analysis would be required to establish if this form of WNV rNS1, which displays mostly immature simple glycans instead of Endo Hf resistant glycans typical of secreted WNV NS1 from infected cells, maintains intact immunological properties.

#### **4.3. Validation of the immunological properties of NS1 proteins**

The use of well-defined sera samples is one of the main requirements to validate recombinant antigens for the development of new flavivirus diagnostic assays. Sera samples can be characterized by molecular and commercial serological tests that have been already validated (i.e., methods recommended by The Pan American Health Organization (PAHO), World Health Organization (WHO), and centers for disease control (CDC)). However, there are many aspects to be considered, including the high degree of cross-reactivity by antibodies developed against flaviviruses (Mansfield et al. 2011; De Paula 2004).

Positivity of a serum sample for a specific flavivirus by molecular methods, or by virus antigen detection, is an indication of a current infection, and it can be followed-up through the detection of IgM and IgG antibodies (Gubler et al. 2017). However, the possibility that the patient was exposed to another flavivirus can be, in many cases, underestimated. It can be because the sample was analyzed by molecular assays after the acute phase when the virus is at undetectable levels, using inappropriate methods, or more commonly, it can be because the screening is not performed. The situation is even more complicated when these sera samples are coming from patients living in endemic regions (Rabe et al. 2016). The probabilities that these patients are exposed to more than one flavivirus are very high, and in most of the cases, they are not diagnosed because the infection is presented without major clinical complications (Jing et al. 2019; Kunze 2012).

Well-defined sera samples to validate the ability of His-tagged NS1 antigens to detect the presence of specific IgM and IgG antibodies can be obtained from immunized mice. Expression of recombinant proteins in mice preserves the native folding and posttranslational modifications required for the development of antibodies that can be used for the validation

of purified antigens (De Almeida et al. 2018; Liu, Wang, and Lu 2016; Rose et al. 2016). Based on this approach, I used sera samples from mice immunized with plasmids encoding V5-tagged NS1 for the validation of the antigens by NS1-based ELISA. Since the IgM antibodies have lower affinity than IgG antibodies because of class switching recombination processes and the affinity maturation (Stavnezer and Schrader 2015), IgM antibodies were detected by incubating sera samples for 1h at 37°C, while the detection of IgG antibodies was done at room temperature. The results obtained by NS1-based ELISA developed in this study showed high sensitivity (100%) and specificity (100%) for the detection of IgM and IgG antibodies from sera of immunized mice.

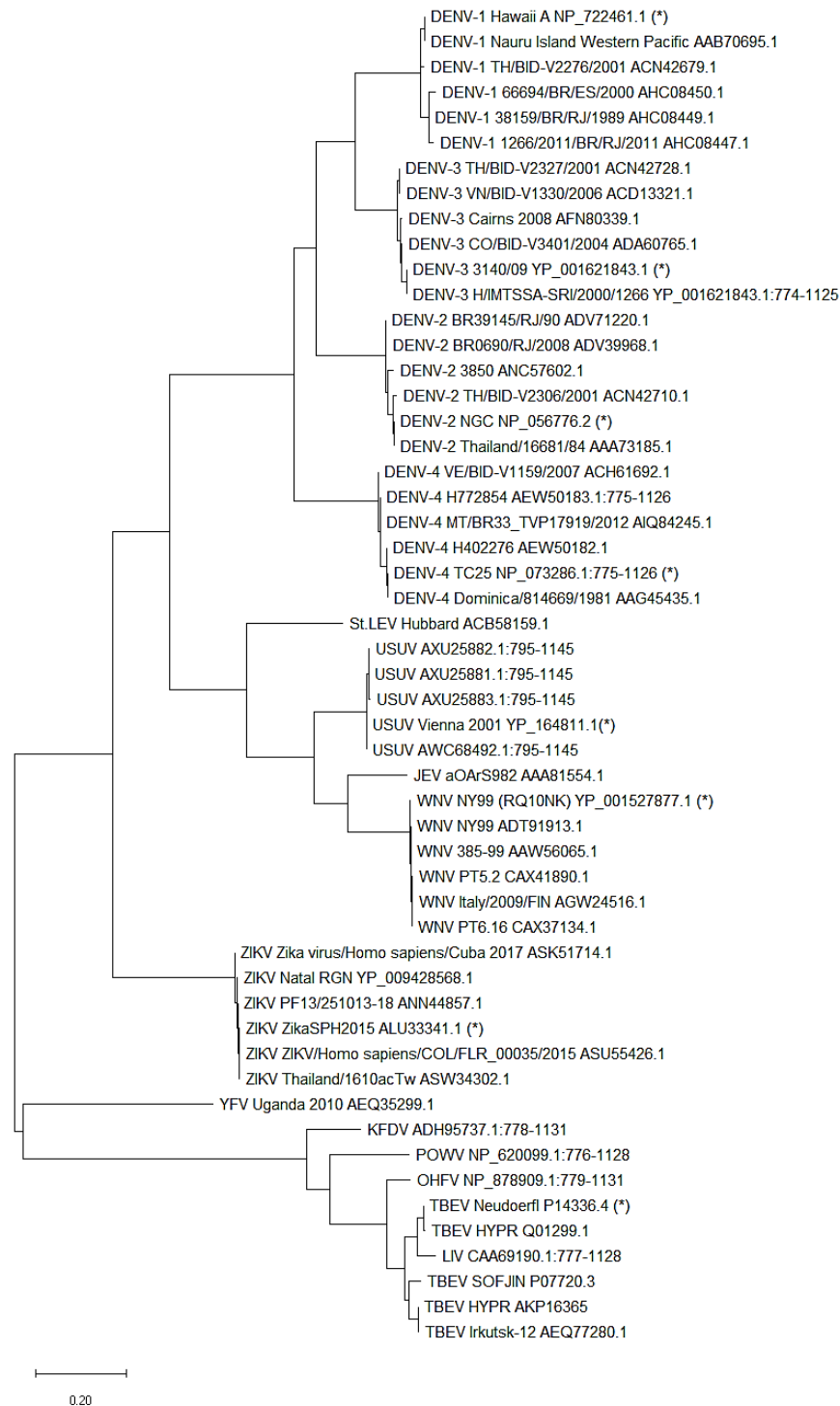
I followed two different schemes for mice immunization. The main difference between both protocols is the moment when the first bleeding was performed after the immunization (day 19 for TBEV, WNV, and ZIKV; day 6 for USUV and DENV1-4). Sera samples were used to compare the sensitivity of IgM detection by NS1-based ELISA when the IgG response is already developed (day 19) or absent (day 6). Previous reports have showed that the depletion of IgG antibodies enhances the sensitivity of IgM antibody detection against envelope protein (Wong et al. 2004), however, I observed comparable sensitivity in the detection of IgM antibodies against NS1 antigen from sera of immunized mice, independently of the levels of IgG antibodies. This finding could suggest that the detection of IgM antibodies against NS1 is not affected by the levels of IgG antibodies. Alternatively, the magnitude of the response in immunized mice may mask subtle differences that could become evident at lower concentrations of antibodies.

In addition to the sensitivity, a test that allows the differential diagnosis of flavivirus infections is required. Commercially produced flavivirus NS1 proteins used in a multiplex NS1-based protein microarray assay have suggested the ability of these antigens to detect IgM and IgG antibodies with limited cross-reactivity from infected patients with WNV, JEV, SLEV, YFV, USUV and DENV1-4 (Cleton et al. 2015). However, commercial NS1 antigens have required some optimization to distinguish flavivirus infections. It includes the combination of several tests (Tsai et al. 2017), a wash step for 5 min with 100 µL urea (4–8 mol/L) after the primary antibody (Tsai et al. 2018), and NS1 blockade-of-binding (BOB) assay that involve the addition of a biotinylated anti-NS1 antibody after serum incubation to calculate the percentage of inhibition (Balmaseda et al. 2017).

In this study, the use of recombinant NS1 antigens purified from the supernatant of transfected HEK-293T cells allowed the detection of IgM and IgG antibodies with high specificity. Recombinant NS1 of TBEV, WNV, ZIKV, and DENV4 detected antibodies from sera samples of mice immunized with the same antigen (100% sensitivity) but not from sera of mice immunized with NS1 of related flaviviruses (100% specificity). These results demonstrate the high specificity of NS1 antigens to detect IgM and IgG antibodies from sera of immunized mice. These results correlate with excellent specificity (92.5%) obtained by the Euroimmune commercial ELISA using the NS1 protein of ZIKV for the detection of IgM and IgG antibodies from infected patients (L'Huillier et al. 2017).

As expected, due to the high degrees of sequence identity between DENV serotypes (Khan et al. 2008), the NS1 antigens of DENV1-3 were less specific for the detection of IgM and IgG antibodies. These antigens did not detect antibodies from sera samples of mice immunized with TBEV, WNV, ZIKV, and DENV4; however, the distinction of DENV serotypes was not clear at high serum concentration. It was more evident in the detection of IgG than IgM antibodies. These results can be explained by the high degree of conserved amino acid sequences between DENV serotypes compared to other related flaviviruses. It is shown in a phylogenetic tree analyzed using the NS1 amino acid sequences of several flavivirus strains (Figure 4.1).

The high degree of conserved amino acid sequences between DENV serotypes can be also observed according to the percentage of identity (the extent to which two amino acid sequences have the same residues at the same positions in an alignment) and positivity (number of amino acids that are either identical or have similar chemical properties) of NS1 protein between different Flaviviruses (Table 4.1). In this table can be appreciated the high degree of identity (above 73%) and positivity (above 84%) between DENV serotypes, while other proteins like TBEV NS1 showed a lower degree of identity (under 43%) and positivity (under 63%) with all NS1 proteins. This data correlates with the results obtained by NS1-based ELISA for the detection of IgM and IgG antibodies from sera of immunized mice.



**Figure 4.1. Phylogenetic tree based on the Maximum likelihood method.** The homology between NS1 amino acid sequences of several flaviviruses was confirmed by the alignment of multiple NS1 amino-acids sequences of TBEV, WNV, ZIKV, USUV, and all DENV serotypes (DENV1-4). A reference strain of each virus used in this study is indicated with an asterisk (\*).

	TBEV	WNV	ZIKV	USUV	DENV-1	DENV-2	DENV-3	DENV-4
TBEV	100/100	42 /63	43/58	41/61	38/55	38/56	38/55	40/56
WNV		100/100	56/70	76/89	51/70	56/74	55/74	54/72
ZIKV			100/100	56/73	54/73	54/73	56/74	54/73
USUV				100/100	51/70	53/73	52/71	52/70
DENV-1					100/100	73/85	80/90	69/83
DENV-2						100/100	74/86	73/84
DENV-3							100/100	74/84
DENV-4								100/100

**Table 4.1. Percentage of identity (red) and positivity (blue) of NS1 protein between different Flaviviruses.** Each NS1 amino acid sequence was aligned using the Protein BLAST tool and compared to related flavivirus.

#### 4.4. Role of the conformation and glycosylation status of NS1 for antibody detection

Western blot under native conditions and deglycosylation analysis of purified NS1 proteins showed high oligomeric status and the presence of post-translational modifications, respectively. These results are comparable to secreted NS1 proteins from infected cells (Macdonald et al. 2005; Muller and Young, 2013). To understand the role of these native characteristics of NS1 proteins on the detection of IgG antibodies, I performed an ELISA assay coating the plates with ZIKV NS1 protein denatured for 10 min at 90°C and/or deglycosylated with PNGase and/or Endo Hf. The results showed that heat denaturation of ZIKV NS1 abolishes the detection of IgG antibodies altogether, indicating that the conformational structure is a requirement for efficient antibody detection. Similarly, the detection of IgG antibodies was drastically affected when ZIKV NS1 protein was treated with denaturing buffer (contains SDS) and/or NP40, detergents required for the deglycosylation assay. These results indicate the relevance of the native structure of NS1 proteins for antibody detection.

Although I observed that some of the components added for the deglycosylation assay (denaturing buffer and NP40) affect the detection of IgG antibodies against ZIKV NS1, the importance of the N-glycans on NS1 proteins for antibody detection remains unclear. I could not have an efficient deglycosylation of NS1 proteins in the absence of denaturing buffer and NP40, as well as, without denaturing the protein by heating the sample.

Several groups have reported that a proper glycosylation status of NS1 proteins is required for NS1 dimerization, stability, and secretion of the NS1 hexamer in mammalian cells (Pryor and Wright 1994; Somnuk et al. 2011). It makes difficult the optimization of strategies to express and purify NS1 proteins that are lacking one or both glycosylation sites, asparagine 130 (N-130) and asparagine 207 (N-207). However, a current publication from Wang and collaborators have demonstrated that NS1 of DENV2, WNV and ZIKV containing a mutation at position N-207 but not at position N-130 can be efficiently purified from the supernatant of transfected HEK-293F suspension cells (Wang et al. 2019). Respect to previous publications, this group has optimized the strategies by expressing NS1 proteins in different cells, adding a specific leader peptide to improve the secretion and following a different approach for protein purification (Wang et al. 2019).

The development of an NS1-based ELISA using NS1 proteins that lack the glycosylation at position N-207 could be an excellent approach to understand the role of high-mannose glycans attached at this site (Pryor and Wright 1994) in the detection of antibodies against NS1 proteins. In addition, since there are some reports where have been shown that in human sera samples there is a vast assortment of antibodies against glycans that can compete for the same antigen than specific antibodies (Muthana et al. 2015), the use of deglycosylated NS1 proteins could help to explain if the high cross-reactivity between some flaviviruses is due to the detection of antibodies against conserved glycans (for example cross-reactive antibodies between DENV serotypes) more than due to the high homology in the amino acid sequences, or it could be due to the combination of both variables.

#### **4.5. Detection of antibodies from human patients**

The novel NS1-based ELISA was used to detect the presence of IgM and IgG antibodies in RT-PCR-confirmed TBEV, WNV, ZIKV, and DENV1-4 positive human sera samples. The same samples were also analyzed by commercial ELISA assays, and the results are discussed in this section.

##### **4.5.1. Comparison of antibody detection from RT-PCR-confirmed TBEV samples**

A total of 100 RT-PCR-confirmed sera samples for TBEV infection revealed a combined sensitivity and specificity of 96% for the detection of IgM and IgG antibodies by NS1-based ELISA. Although, comparable results were obtained in all the samples analyzed by commercial



ELISA and NS1-based ELISA assays, seven samples from the convalescent phase of TBEV infection were IgM negative by NS1-based ELISA while they were considered positive by commercial ELISA. It could be due to lower sensitivity of the NS1-based ELISA; however, considering that these samples were collected during the convalescent phase (from two months after the onset of symptoms), these results could suggest the detection of false positives by commercial ELISA. The detection of IgM antibodies is considered a marker of acute TBEV infection and can also be detected for several months (Dippe 1983). However, the potential of IgM false-positive results has also been suggested due to possible IgM persistence, heterologous flavivirus infections, and vaccine-induced antibodies (Stiasny et al. 2012).

Importantly, previous reports have demonstrated that the immune response developed against TBEV can neutralize related flaviviruses such as Louping ill virus, WNV, and DENV2 (Klaus et al. 2014; Mansfield et al. 2011). It could indicate that antibodies generated against other flaviviruses could also react against TBEV antigens resulting in false positive results. In particular, the TBEV sera samples used in this study are from Slovenian patients, an endemic region for TBEV (Saksida et al. 2018), and with an increasing number of WNV cases (Holt 2018). Furthermore, the commercial Enzygnost anti-TBE ELISA used for the diagnosis is based on plates coated with inactivated TBEV. It means that the principal antibodies detected by this assay are directed against envelope protein, an antigen that has been characterized for the presence of a conserved fusion loop between flaviviruses (Crill, and Chang 2004; Lai et al. 2008; Seligman 2008), and cross-reactive antibodies profoundly recognize it (Rockstroh et al. 2017; 2018; Rizzo et al. 2019). Taken together, the differences obtained between both ELISA assays could suggest the detection of cross-reactive IgM antibodies by commercial ELISA and emphasize the use of NS1 antigen for the specific IgM detection in the diagnosis of TBEV.

The extensive comparison by testing TBEV-infected sera samples from first, second, acute and convalescent phases simultaneously with commercial ELISA and NS1-based ELISA assays showed a high degree of correlation between both assays for the detection of IgM antibodies. However, a low correlation was obtained for the detection of IgG antibodies. Although 89/89 IgG positive and 11/11 IgG negative TBEV-confirmed sera specimens from first, second, acute and convalescent phases showed high or low P/N ratio values, respectively, by NS1-based ELISA, variable values by commercial ELISA were obtained (range, 5.7 to 883 U/mL). High variability between commercial ELISA assays for the detection of IgG antibodies has been already reported. The most current publication from Ackermann-Gäumann et al. reported

discrepant results in 37.2% of all samples analyzed (876) by four different commercial ELISA assays (the Enzygnost Anti- TBE/FSME Virus Siemens; the Anti-FSME/TBE Virus ELISA, Euroimmun; the Anti-FSME/TBE Virus ELISA “Vienna”, Euroimmun; and the RI- DASCREEN FSME/TBE IgG EIA assay, R-Biopharm) (Ackermann-Gäumann et al. 2019). Unlike commercial assays, the performance of the NS1-based ELISA showed consistent results. It supports the use of this assay for the detection of both IgM and IgG antibodies against TBEV.

#### **4.5.2. Differential diagnosis of TBEV infected patients**

TBEV vaccine breakthrough can occur in several patients due to lack of complete seroconversion after vaccination, non-responsiveness to the antigen present in the vaccine, inappropriate storage of the vaccine that leads to loss of antigenicity or infectivity in the case of live vaccines (Stiasny, Holzmann, and Heinz 2009). Therefore, the differential diagnosis between vaccinated and naturally infected patients with TBEV is crucial to discriminate vaccine breakthrough cases (Sendi 2017). However, the commercial ELISA assays available for the diagnosis of TBEV are mainly based on the detection of antibodies against envelope protein, an antigen that is also present in TBEV vaccines and is highly cross-reactive between flaviviruses (Albinsson et al. 2018; Rockstroh et al. 2018). Independently if the person has been vaccinated or naturally infected with TBEV will develop antibodies against envelope protein, and it cannot be differentiated using the available commercial ELISA assays (Veje et al. 2018).

As expected, the detection of IgM and IgG antibodies by the commercial Enzygnost anti-TBE ELISA did not distinguish between TBEV vaccinated, natural infected, or vaccine breakthrough (VBT) sera samples. Independently of the sample, all of them were IgG positive. Meanwhile, the number of IgM positive samples was higher from naturally infected patients (10/10) than from vaccinated people (7/10). This data correlates with previous reports where have been demonstrated that the level of IgM antibodies in VBT sera samples is very low and in many cases, at undetectable levels (Stiasny, Holzmann, and Heinz 2009). Importantly, results obtained by commercial ELISA indicate that three sera samples from YFV vaccinated people were IgG positive for TBEV, while they were negative when analyzed by NS1-based ELISA. It supports the need for specific assays to distinguish TBEV infections from other flaviviruses.

So far, serological assays aiming to detect antibodies against NS1 of TBEV have not been produced in any system. Here, I developed and optimized an NS1-based ELISA to distinguish not only TBEV from other related flaviviruses but also the differential diagnosis between

different groups of TBEV sera samples. First, I demonstrated that three sera samples of individuals vaccinated against YFV did not react with TBEV NS1 antigen suggesting the ability of this assay to differentiate flavivirus infections, as was also shown in the validation of the antigens using sera of immunized mice. Secondly, the detection of IgM and IgG antibodies based on TBEV NS1 allowed the differential diagnosis between TBEV vaccinated, naturally infected, and VBT sera samples. Contrary to the results obtained by commercial assays in this study and other publications, the number of positive IgM VBT samples and the level of IgM antibodies from the same group showed higher sensitivity of the NS1-based ELISA compared to commercial ELISA. Together, the results support the usefulness of the NS1-based ELISA for differential diagnosis of TBEV with high sensitivity and specificity.

#### **4.5.3. Detection of IgM and IgG antibodies from RT-PCR-confirmed WNV, ZIKV, and DENV1-4 sera samples**

NS1-based ELISA for virus-specific IgM/IgG detection performed well also for WNV, ZIKV, and all DENV serotypes. The results were compared to those obtained by commercial assays based on NS1 protein in case of ZIKV (Eurimmune NS1 ELISA), and ELISA assays based on envelope protein for the detection of antibodies against WNV (Focus Diagnostics) and all four DENV serotypes (plates coated with envelope protein of DENV2, NovaTec immunodiagnostic GmbH).

##### **4.5.3.1. WNV antibody detection**

According to the total number of positive samples, the sensitivity of the detection of IgM antibodies against WNV by NS1-based ELISA (10/16) and commercial ELISA (12/16) assays was comparable. As expected, some of the samples were IgM negative because they were collected during different phases after WNV infection, including samples collected very late after the convalescent phase when IgM levels are very low or at undetectable levels (Busch et al. 2008). Importantly, high sensitivity and specificity of the Focus Diagnostics assays for the detection of WNV have been confirmed in several studies (Hogrefe et al. 2004; Malan et al. 2004; Rawlins et al. 2007; Welch, Anderson, and Litwin 2008). It is supported by the background subtraction protocol (a protocol where the absorbance of a sample composed only by buffers is subtracted to the serum sample that is being analyzed) to remove unspecific reactivity and avoid false-positive results. A slightly higher sensitivity of the NS1-

based ELISA (12/16) was observed for the detection of IgG antibodies when the results were compared to those obtained by commercial ELISA assays (9/16).

The results demonstrate comparable sensitivity and specificity between the NS1-based ELISA and the focus diagnostics ELISA, one of the most used commercial assays for the diagnosis of WNV (Welch, Anderson, and Litwin 2008). Besides, the detection of specific IgG antibodies against NS1 protein showed higher sensitivity than the commercial assay based on envelope protein. These results suggest the usefulness of the NS1 antigen for the diagnosis of WNV with equal or even higher sensitivity than the commercial assay used in this study.

#### **4.5.3.2. ZIKV antibody detection**

The Euroimmune ZIKV NS1 assay is the only test commercially available for the diagnosis of flavivirus infections based on the detection of antibodies against NS1. This assay has been shown to distinguish ZIKV infections from other related flaviviruses such as DENV with high IgM/IgG specificity (99.8%) in a study including 1015 samples (Steinhagen et al. 2016); The same study and others have also reported low sensitivity for the detection of IgM antibodies (58.8%) compared to IgG detection (88.2%) (L'Huillier et al. 2017; Steinhagen et al. 2016). In terms of the total number of positive samples, comparable sensitivity from 15 RT-PCR confirmed ZIKV samples were obtained using the Euroimmune assay to detect IgM (11/15) and IgG (14/15) antibodies. However, higher sensitivity (15/15) for IgM and IgG detection was obtained by NS1-based ELISA developed in this study.

In both assays, sera samples were pre-incubated with GullSORB reagent (Meridian Bioscience Inc.) to remove class IgG antibodies and rheumatoid factor from the sample that could interfere with IgM detection and give the unspecific signal, respectively. In both assays, sera samples were also incubated at 37°C for 1 hour. Apart from the differences in the protocols to produced and purified ZIKV NS1, the main difference between both assays is the serum dilution used for antibody detection. Euroimmune assay was performed diluting the samples 1:100 according to the manufacturer's instructions, while 1:20 dilution was used for NS1-based ELISA assay. It could influence the sensitivity of the assays; however, an analysis expanding the number of samples is required. The most important output of this analysis is the validation of the ZIKV NS1 antigen for the detection of antibodies from sera of infected patients.

#### **4.5.3.3. DENV1-4 antibody detection**

The diagnosis of DENV is mainly made to detect the presence of IgM and IgG antibodies by ELISA assays. Generally, the performance of these assays shows good sensitivity for the detection of IgG antibodies; however, the low sensitivity of the available commercial diagnostic assays for the detection of IgM antibodies is one of the main difficulties in the diagnosis of DENV (Lee et al. 2019). I noticed similar results when DENV RT-PCR-confirmed sera samples were analyzed using the NovaTec immunodiagnostic GmbH assay. Low IgM sensitivity was detected in sera samples from all four DENV serotypes. It was more evident for DENV4; only 3 out of 8 samples were IgM positive. On the contrary, when the same samples were analyzed by NS1-based ELISA, I obtained 100% sensitivity for all DENV serotypes. It was done by reacting each serotype-specific serum sample with the respective serotype-specific NS1 antigen.

One of the main reasons that explain these results is due to the use of a single antigen to detect all four DENV serotypes. Notably, the NovaTec immunodiagnostic GmbH assay is based on the detection of IgM and IgG antibodies against the envelope protein of DENV2, one of the most spread DENV serotypes (Messina et al., 2014). It has been reported that the envelope protein of DENV2 is highly cross-reactive between flaviviruses. However, the probabilities of missing the detection of specific serotypes are underestimated. As discussed during the validation of NS1 antigens to detect DENV specific antibodies, not all the antigens detect cross-reactive antibodies from other DENV serotypes, as demonstrated by using NS1 antigen of DENV4.

#### **4.6. Identification, isolation, and seroprevalence of USUV from blood donors in North-Eastern Italy**

The Northern Eastern region of Italy is endemic for flaviviruses such as tick-borne encephalitis virus (TBEV) and west nile virus (WNV) (Calzolari et al. 2015; Rezza et al. 2013). Importantly, WNV is well-known to be transmitted by blood transfusion (Pealer et al. 2003). Therefore, blood donors are routinely screened for WNV infection. USUV co-circulates with WNV in many European countries and share host and vector species (Zannoli and Sambri 2019). The year 2018 showed a dramatic increase of USUV and WNV infections in Europe with increased transmission to humans (Aberle et al. 2018). Furthermore, circulation of USUV in mosquitoes has been described in the region (Calzolari et al. 2015). However, in

this work for the first time, the presence of USUV transmission to humans is being documented by isolation and a retrospective serological analysis. Two samples of serum from 44 blood donors were confirmed as new infections with USUV (IgM and IgG seroconversion to USUV), one also by nucleic-acid test (NAT) and virus isolation.

USUV was initially isolated in Africa in 1959, and it has been continuously circulating with several introductions in Europe through migratory birds since the first documented outbreak in Vienna 2001 (Ashraf et al. 2015; Cheng et al. 2018; Weissenböck et al. 2003). Therefore, several distinct lineages of USUV (Europe 1-5) currently co-circulate (Cadaru et al. 2017; Roesch et al. 2019). Sequencing analysis of the USUV isolate from the blood donor in North-Eastern Italy indicates that it belongs to the Europe 1 lineage. A lineage that has been circulating in Italy and neighboring countries at least since 2009 (Manarolla et al. 2010; Engel et al. 2016; Cadaru et al. 2017)

Transfusion-transmitted USUV infection has not been reported so far. However, USUV-infected donations in the European Union blood supply have been detected during routine screening of blood donations for WNV RNA (Aberle et al. 2018; Bakonyi et al. 2017; Cadaru et al. 2017). USUV antibodies in blood donors had also been detected (Allering et al. 2012; Gaibani et al. 2012). The prevalence of USUV among blood donors is not fully established also because there is no requirement to screen blood donors for USUV RNA. Assessing the risk of USUV transmission through blood transfusion is, therefore, crucial. The cross-reactivity of WNV NAT with USUV can contribute to the detection of these flaviviruses in donated blood (Aberle et al. 2018; Bakonyi et al. 2017). However, WNV NAT-reactive donations should undergo virus-specific confirmatory tests (Domanović et al. 2019).

To monitor the seroprevalence of USUV in North-Eastern Italy, I performed a retrospective analysis in a cohort of 139 sera samples from blood donors. The results showed a high seroprevalence (34.5%) of IgG to USUV by the NS1-based ELISA. Since, available commercial assays for USUV are scarce (there is only one test for IgG detection) and exploit mostly virion components as antigens that suffer from broad antigenic cross-reactivity of anti-flavivirus antibodies (Koraka et al. 2002; Singh et al. 2018), the results of IgG detection from only 44 sera samples were compared to those obtained by commercial Euroimmune ELISA (plate coated with USUV envelope protein).

The use of USUV NS1 as an alternative antigen to virion proteins for the detection of IgG

antibodies against USUV showed Higher sensitivity and specificity compared to the commercial ELISA. High sensitivity and specificity of USUV NS1 antigen have also been proven by protein microarray using USUV NS1 commercial antigens (Cleton et al. 2015). In addition, the same assay allowed the detection of IgM antibodies with high sensitivity and specificity. It is the first report of IgM detection by ELISA using purified NS1 antigens due to the lack of commercial assays. Although cross-reactivity cannot be completely ruled out, the results may also indicate co-infections with WNV and USUV occurring in the same patients. This hypothesis is sustained by the fact that viruses co-circulate in the same area and share hosts, vectors and seasonality. Furthermore, recent report identified human cases with co-infection by WNV and USUV in the same individual (Aberle et al. 2018; Faggioni et al. 2012; Vilibic-Cavlek et al. 2014).

In conclusion, the area of North-Eastern Italy is endemic for USUV virus, and the development of a new NS1-based ELISA test not only allowed the study of the high seroprevalence of USUV infection in blood donors of the region but also provided a tool for further epidemiological and clinical use.

#### **4.7. Seroprevalence of flavivirus in Borno State, North-Eastern Nigeria**

Many countries in Africa, including Nigeria, are endemic for emerging and re-emerging infectious diseases that cause similar clinical manifestations. Febrile-illness patients are usually treated considering critical human pathogens that are recognized to be circulating in the region such as malaria, YFV, and typhoid. Although mosquito-borne viruses like Chikungunya, ZIKV, DENV have been reported in Nigeria four to six decades ago (Moore et al. 1975), the real situation of flaviviruses is underestimated mainly due to under-reporting or lack of awareness (Oluwayelu, Adebisi, and Tomori 2018). The establishment of appropriate diagnostic facilities and availability of reagents for arboviral infections in Nigeria, and in Africa in general, is therefore crucial for active surveillance towards the prevention and control of these infections.

Towards this aim, the NS1-based ELISA developed in this study was implemented for the surveillance and diagnosis of flaviviruses in febrile-illness patients who visited the University of Maiduguri Teaching Hospital (UMTH) Borno-state, Nigeria. This study was performed for the specific detection of IgG antibodies to TBEV, WNV, ZIKV, USUV, and DENV1-4). 93.5% of the sera samples had NS1 IgG antibody for at least one flavivirus, while only 6.5% of the

patients were negative for all the flaviviruses tested. In addition, the samples were screened for flaviviral RNA detection by hemi-nested RT-PCR and sequencing analysis. The specific detection and seroprevalence for each virus is discussed as follows below.

#### 4.7.1. Seroprevalence of TBEV

The distribution of tick-borne encephalitis virus (TBEV) have been reported in large areas of Europe and Asia (Hayasaka et al. 2001). According to the world health organization, the highest incidences of TBEV are published in the Baltic States, the Russian Federation, and Slovenia. Although TBEV has not been reported in Africa, the potential of the geographic distribution of TBEV vectors (in particular *Ixodes Ricinus* ticks) has been reported in some countries in North Africa including Tunisia, Algeria, and Morocco as a result of the influences of climate change (Alkishe, Peterson, and Samy 2017). The presence of *Ixodes* ticks has also been detected in migratory birds between Africa and Europe (Pascucci et al. 2019).

These previous publications provide some insights to explain the results obtained by sequencing analysis of 2 positive hemi-nested RT-PCR samples, and the high seroprevalence (53.4%) of IgG antibodies against TBEV found in Borno-state, Nigeria. TBEV is mainly transmitted by the bite of an infected *Ixodes Ricinus* or *Ixodes persulcatus* ticks in Europe and Asia. It could be possible that these ticks carrying TBEV are also circulating in the region, but they have not been detected because of the lack of proper studies. According to the results obtained by hemi-nested RT-PCR and sequencing analysis, we found two possible human sera samples positive for TBEV. However, the sequencing analysis must be confirmed, amplifying a more significant region to validate the results.

Another possibility could be due to the transmission of TBEV by a different tick vector. It could be possible since the presence of flavivirus RNA has been detected in *H. marginatum* and *Amblyomma sp.* ticks collected from migratory birds between Africa and Europe (Pascucci et al. 2019), and also because the presence of different ticks species mainly belonging to the family *Ixodidae* have been found in a study performed in the same region where the human sera samples used in this study were collected (Musa et al. 2014).

Finally, a third possibility to explain the seroprevalence against TBEV is due to the detection of antibodies developed against a different tick-borne virus. TBEV is a member of the TBEV serocomplex, which includes genetically close viruses such as Omsk hemorrhagic fever virus,



Kyasanur Forest disease virus, Powassan virus, and Louping ill virus. As shown in figure 4.1, the similarities of NS1 sequences of these viruses could suggest that infections with these viruses induce an antibody response that could cross-react with TBEV NS1. The P/N ratios obtained from this analysis showed a strong signal in several samples indicating specific detection of antibodies against TBEV-NS1. However, some of them were not very strong. It could depend on the levels of antibodies developed against TBEV but also support the idea of the detection of cross-reactive antibodies from related tick-borne viruses.

#### **4.7.2. Seroprevalence of WNV**

The presence of west Nile virus in Africa has been reported since the first isolation in 1937 in Uganda (Smithburn et al. 1940), followed by a rapid spread around the globe, including one of the most well-documented outbreaks in New York in 1999 (Hayes 1999). Although WNV has been continuously reported in several African countries since the first identification (Chancey et al. 2015), a few surveillance studies have reported the current situation.

In this study, 11 positive samples for flaviviral RNA by hemi-nested RT PCR were confirmed for WNV by sequencing analysis indicating the circulation of the virus in the region. In addition, high seroprevalence (85.3%) of IgG antibodies was observed by NS1-based ELISA. It is not surprising since WNV has already been reported in mosquitos and febrile-illness patients from the same region with suspected malaria infection (Baba et al. 2006). More recent publications have also demonstrated the presence of IgM antibodies by ELISA (Kolawole, Adelaiye, and Ogah 2018) or neutralizing antibodies by plaque reduction neutralization test (Baba et al. 2013). However, other studies have revealed no serological evidences against WNV in Blood Donors in South Western Nigeria (Opaleye et al. 2014), or low IgM seroprevalence (7.5%) of WNV in a study including 200 sera samples of febrile-illness patients from Kwara in Northern Nigeria (Kolawole, Adelaiye, and Ogah 2018).

Based on the results presented in this study, the absent or low seroprevalence of WNV observed in current publications could be due to the lack of proper screening assays for the diagnosis and surveillance of WNV in the country. It could be improved by the implementation of serological assays based on the NS1 protein of WNV that allows specific antibody detection. It is an important aspect that has to be considered due to the high prevalence of arboviruses in the region (Baba et al. 2006; 2009).

#### 4.7.3. Seroprevalence of ZIKV

High seroprevalence of IgG antibodies against NS1 of ZIKV (74.6%), as well as, circulation of ZIKV was demonstrated in this study using sera samples collected in North-Eastern Nigeria. All the samples were analyzed by NS1-based ELISA, and hemi-nested RT-PCR, followed by sequencing, respectively. Although ZIKV has not been reported in the region (Baba et al. 2013), the circulation of ZIKV in Africa has been reported since 1947 when the virus was isolated in Uganda from the serum of a febrile sentinel rhesus monkey (Dick et al. 1952). The isolation of ZIKV from humans is not completely clear since some reports indicated it was in 1954 from a 10-year-old Nigerian female coinfecting with malaria (Macnamara et al. 1954), but others have reported the first case of confirmed human ZIKV infection occurred in Uganda in 1962–1963 (Gelfand et al. 1964). Independently whether the Nigerian isolation was zika or not, subsequent studies demonstrated the circulation of the virus in several regions in Nigeria (Moore et al. 1975; Fagbami et al. 1978; 1979). However, epidemiological information about the current situation of ZIKV in Nigeria and Africa, in general, has not been reported for many years.

Only two current studies are found in the literature where have been studied the seroprevalence of ZIKV in Nigeria. The first one (6.2% seroprevalence) is a retrospective study including 188 samples collected between 2004 and 2016 from a cohort of Nigerian HIV treatment patients (Herrera et al. 2017), while the second and more recent study (6% seroprevalence) includes a group of 468 individuals mostly comprised of pregnant women in North Central Nigeria (Mathé et al. 2018). The first study was based on the detection of IgM antibodies by MyBioSource ELISA that has a high risk for the detection of false positives, while the second one was done using the Euroimmune NS1-based ELISA that is highly specific. Independently of the assays, they generally showed as a largely ZIKV immunologically naïve population that does not correspond with the results obtained by NS1-based ELISA developed in this study. It could be because ZIKV is restricted to the North-Eastern region, but most probably, it is due to the lack of proper surveillance and epidemiological studies.

Based on the high sensitivity and specificity of the ZIKV NS1 antigen to detect IgM and IgG antibodies from sera of immunized mice and RT-PCR confirmed patients, the differences in the seroprevalence between the studies could mainly be due to the region where the

samples were collected, the selection of the cohorts and management of the samples. Importantly, the first study included samples collected for 12 years, while in the second study, the samples were sent to be processed in Germany. It could be possible that an inappropriate transport and storage of the samples have affected the analysis. Also, the first study was done in a cohort of Nigerian HIV treatment patients, a group of patients that probably could develop a different immune response. In any case, the high seroprevalence obtained by NS1-based ELISA emphasizes the importance of ZIKV surveillance in Nigeria and other countries in Africa for proper diagnosis, especially in febrile illness patients that could be misdiagnosed with other endemic infectious diseases such as malaria and typhoid.

#### 4.7.4. Seroprevalence of USUV

Comparable to other flaviviruses mentioned above that were discovered in Africa, USUV was isolated for the first time in South Africa from *Culex naevi* mosquitoes (Williams et al. 1964), followed by the detection in many other African countries (Nikolay et al. 2011). However, limited information is found in the literature about the isolation of the virus from infected humans. Many reports indicate the first human case of USUV in a patient presenting fever and rash in the Central African Republic in 1981, followed by a case in Burkina Faso in 2004 (Nikolay et al. 2011). The significant attention of USUV increases when the virus caused an extensive die-off of several species of wild birds in Austria 2001 (Weissenböck et al. 2002; 2003) and neurological complications in two immune-compromised infected patients in Italy (Pecorari et al. 2009). After this, many reports have shown the circulation of USUV in many European countries, and many surveillance programs have been implemented to control the expansion in naïve areas (Ashraf et al. 2015).

Although a single study had reported the seroprevalence of USUV infections in Nigeria more than forty years ago when the antigenic relationships of WNV with other flaviviruses were analyzed (Odelola et al. 1975), I found 64.5% IgG seroprevalence in febrile-illness patients from North-Eastern Nigeria. USUV is mainly transmitted by *Culex* mosquitos that are well-known to be present in Nigeria. A study performed in 2013 demonstrated the circulation of several species of mosquitoes, including 7 *Culex* species with the highest abundance in Benin City, in southern Nigeria (Aigbodion and Uyi 2013). Importantly, two additional studies collecting mosquitos in different regions from North-Eastern Nigeria have demonstrated the prevalence of more than 80% of *Culex species* in the area. This information emphasizes the

importance of the implementation of surveillance programs to understand the burden of USUV in the region. It could be done by the NS1-based ELISA developed in this study. It is a cheaper and less complicated assay that can also be applied for the detection of IgM antibodies, an assay that is not available in the market.

#### **4.7.5. Seroprevalence of DENV1-4**

Dengue virus, including all four closely related serotypes (DENV1-4), has a global distribution and causes more than 390 million infections per year (Bhatt et al. 2013). It has been estimated that 3.9 billion people, in 128 countries, are at risk of infection with dengue virus (Brady et al. 2012). Based on a current meta-analysis performed from 2000 to 2019, Africa was identified with a high prevalence of DENV. This study showed not only high prevalence in populations presenting fever but also in apparently healthy individuals where 15.6% and 3.5% turn on positive for the detection of antibodies and RNA, respectively, (Simo et al. 2019). This study suggests the importance of surveillance programs, even in those countries where sporadic cases have been reported.

Initially, the first cases of DENV in Nigeria were isolated between 1964-1968, and they were identified as dengue virus type 1 and type 2 (Carey et al. 1971); since then, the circulation of all four DENV serotypes have been reported (Baba et al. 2009; Fagbami and Onoja 2018). Although there are not many reports of the burden of dengue virus infections in Nigeria, the results obtained by sequencing analysis and NS1-based ELISA showed the circulation of DENV1 and DENV4, and the highest seroprevalence between all the flaviviruses tested in this work. This evidence highlights the need to increase dengue surveillance in Nigeria to reduce the risk of hemorrhagic fever, one of the significant causes of morbidity and mortality in the country (Faduyile et al. 2017).

Taken together, the identification of several flaviviruses by sequencing analysis (still ongoing) and the high seroprevalence observed by NS1-based ELISA stress the burden of flaviviruses in the region. It could be also similar in other regions in Nigeria and African countries where the NS1-based ELISA for the diagnosis and surveillance programs could be implemented. It could be very important in low-income countries where the climatic conditions, circulation of several flaviviruses, presence of other hemorrhagic infectious diseases, and inappropriate healthcare settings make the identification of the infecting agent more complex.

## 5. CONCLUSIONS

- The recombinant NS1 protein of TBEV, WNV, ZIKV, USUV, and all four DENV serotypes was efficiently purified with high yield from the supernatant of transiently transfected HEK-293T cells treated with sodium butyrate.
- The purified recombinant NS1 proteins preserved the native conformational and glycosylation status characteristic of NS1 protein produced in mammalian cells.
- The use of recombinant NS1 of TBEV, WNV, ZIKV, and DENV4 allowed the detection of IgM and IgG antibodies with 100% sensitivity and specificity from sera samples of mice immunized. The NS1 antigens of DENV1-3 showed comparable sensitivity with the detection of some cross-reactive IgM and IgG antibodies between DENV serotypes.
- The native conformational structure of the ZIKV NS1 protein is a requirement for efficient IgG antibody detection from sera of mice immunized with ZIKV.
- The NS1-based ELISA allowed the detection of IgM and IgG antibodies from sera samples of RT-PCR confirmed patients with higher sensitivity compared to standard commercial assays. Limited to TBEV, for which a vaccine is available for humans, serology based on NS1 protein can distinguish vaccine breakthrough cases of infection.
- This study identified the isolation of the first case of USUV from a blood donor in the Friuli Venezia Giulia Region of North-Eastern Italy. NS1-based ELISA confirmed it in follow-up samples. Also, the implementation of the NS1-based ELISA in a retrospective study identified North-Eastern Italy as an endemic for USUV with implications for the screening of transfusion blood.
- The implementation of the NS1-based ELISA in febrile-illness patients from North-Eastern Nigeria demonstrated a high seroprevalence of flaviviruses in the region. The highest seroprevalence was obtained for DENV1-4, followed by WNV, ZIKV, USUV, and TBEV.

In conclusion, the results presented and discussed in this thesis showed that the NS1-based ELISA is a sensitive and highly specific test for the detection of IgM/IgG antibodies, indicating its potential use in serodiagnosis and surveillance studies. The NS1-based ELISA could also be transferred to a point of care test for a syndrome-based multi-parametric diagnosis of flavivirus, a necessary test, especially in those regions where more than one flavivirus is circulating.

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## 6. REFERENCES

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